MOLECULAR AND IMMUNOLOGICAL CHARACTERIZATION OF KENTUCKY BLUEGRASS (*Poa pratensis*) GROUP IX ALLERGENS

BY

LEI ZHANG

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

DEPARTMENT OF IMMUNOLOGY
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TABLE OF CONTENTS

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LIST OF ABBREVIATION

Ag	antigen
AP	alkaline phosphatase
APC	antigen presenting cell
Amp	Ampicillin
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BDF1	B_6D_2F1 mice
BSA	bovine serum albumin
β-gal.	β-galactosidase
CD	cluster of differentiation antigen
cDNA	complementary DNA
CFA	complete Freund's adjuvant
DS	dextran sulfate
ELISA	enzyme-linked immunosorbent assay
FP	fusion protein
HLA	human leukocyte antigen
IFN	interferon
IL	interleukin
IPTG	iso-propyl-β-thiogalactoside
kDa	kilo dalton
KBG	Kentucky Bluegrass
LB medium	Luria-Bertani medium
MHC	major histocompatibility complex
mRNA	messenger RNA
MW	molecular weight

Mab	monoclonal antibody
NTB	*p*-nitro tetrazolium chloride
OD	optical density
PBS	phosphate buffered saline
PBST	PBS Tween-20 buffer
PCA	passive cutaneous anaphylaxis
Poa p	*Poa pratensis*
pWR590	pWR590 plasmid vector
rDNA	recombinant DNA
rKBG	recombinant KBG protein
SDS-PAGE	sodium dodecyl sulphate poly-acrylamide gel electrophoresis
Th	helper T cells

LIST OF TABLES AND FIGURES

PAGES

Chapter IV

Chapter V

Chapter VI

ABSTRACT

Previously, screening of a λgt11 library established with mRNA of Kentucky Bluegrass (KBG) pollen using human IgE antibodies led to the isolation of three full-length cDNA clones, KBG30, KBG41 and KBG60. For further immunochemical characterization of the cloned allergens, one of the cDNA clones, KBG60, and its fragments were generated and synthesized in large amounts in *E. coli* using gene expression procedures. According to the sequence analysis, one fragment of the recombinant KBG60 (rKBG60), viz. rKBG8.3, was found to span the conserved region of all the three cDNA clones. Antibodies raised against the rKBG8.3 were used as a probe to identify the corresponding native proteins in KBG pollen extract. It was clearly shown by western blotting analysis that three protein bands with molecular weight 28KDa, 32KDa and 34KDa were recognized by the antibodies. This antibody probe and human IgE antibodies were further employed to study the antigenically and allergenically cross-reactive components in different species of grass pollens. Thus, a new group of isoallergens encoded by the cDNAs were identified from Kentucky Bluegrass pollen, designated as *Poa p* IX isoallergens in accordance with the existing International Allergen Nomenclature system. Subsequently, the existence of a family of proteins in a variety of grass pollens related to *Pao p* IX allergen was demonstrated.

The rKBG60 allergen and its four overlapping fragments, rKBG8.3, rKBG60.1, rKBG10 and rKBG60.2, were synthesized in *E. coli*

by employing a plasmid expression vector, pWR950.1. The vector contains a partial *Lac Z* gene coding for a truncated β-galactosidase comprising the N-terminal 590 amino acids. Therefore, each of the recombinant polypeptides was synthesized as a fusion protein.

It was demonstrated that the recombinant allergenic polypeptides synthesized in our gene expression system were able to bind to the murine and human IgE antibodies specific to KBG pollen allergens. Thus, the antibody-binding epitopes of the rKBG60 allergen were analyzed with its overlapping recombinant fragments. The results indicated that, although antibody-binding epitopes were distributed all over the molecule, the major epitopes of the rKBG60 allergen were located on its C-terminal region, represented by the recombinant polypeptide, rKBG8.3.

For further analysis of the antibody-binding epitopes, overlapping decapeptides fixed on polypropylene pins and the 20 residue peptides were synthesized according to the amino acid sequence deduced from the nucleotide sequence of the KBG60 cDNA clone. Binding studies of the peptides with the murine antisera raised against the recombinant allergens indicated that at least 13 antibody-binding epitopes were present on the rKBG60 allergen. Some of these peptides, which were recognized by murine antibodies, were also able to react with human IgG and human IgE antibodies. At least 6 human IgG and 4 IgE epitopes were thus defined. Human IgE antibodies showed the most restricted binding patterns with the peptides. The peptides recognized by human IgE were found mostly

located at the C-terminal region of the rKBG60, consistent with the results observed with the recombinant polypeptides. Furthermore, by induction of the allergen-specific antibodies by immunization of the mice with the synthetic peptides, it was demonstrated that the majority of the antibody-binding peptides also possessed T cell epitopes.

The recombinant *Poa p* IX proteins were further studied in terms of their abilities to induce IgE antibodies *in vivo*. The results indicated that the recombinant allergenic polypeptide, rKBG8.3, had the capacity of inducing KBG-specific IgE antibodies in BDF1 mice. The IgE antibodies induced by the rKBG8.3 were found to remain in circulation in mouse sera for at least 60 days without apparent decrease in their titers and were responsible for passive anaphylactic responses triggered by allergens, not only of KBG pollen, but also of other species of grass pollens. From these results, it was inferred that the mouse model constituted a useful animal model for studies of the allergenicity of the recombinant allergens *in vivo*.

Since the rKBG8.3 was composed of two components, a non-allergenic truncated β-galactosidase and an allergenic *Poa p* IX polypeptide, this fusion protein was regarded as a recombinant antigen-allergen chimeric (RAAC) protein and its immunogenicity was investigated. It was found that the immune responses induced by the RAAC protein could be classified into two categories: Th1-like responses to β-galactosidase and Th2-like responses to the *Poa p* IX allergens. This observation was confirmed by using the individual

components, i.e., the truncated β-galactosidase and the allergenic *Poa p* IX polypeptide. In addition high titers of IgG1 antibodies were induced by both antigens, the β-galactosidase induced mainly IgG2a antibodies, whereas the allergenic *Poa p* IX polypeptide induced primarily IgE immune responses, when these antigens were injected in conjunction with dextran sulphate for immunization of the mice. Modulation of the IgE immune responses to the allergenic *Pao p* IX polypeptide could be achieved only when the RAAC protein, but not the allergenic *Poa p* IX polypeptide, was used in complete Freund's adjuvant for immunization. Thus, these results suggest a possible modality to change the IgE responses into IgG2a responses to the allergens by linkage of the allergens onto the non-allergenic protein and usage of the proper adjuvant for immunization.

CHAPTER I

INTRODUCTION

SCOPE OF THE THESIS

Allergic diseases are common immunological disorders, afflicting about 20% of the total population. The incidence of these diseases has been increasing in both the industrialized and developing countries. IgE immune responses, leading to immediate hypersensitivity in atopic individuals and experimental animal models, are characterized by the induction of IgE antibodies, and initiated by the interaction of the two complementary molecules, the allergenic proteins and the corresponding IgE antibodies. IgE immune responses have been considered as a major component of the immune system against helminth and nematode infections(1,2). However, similar responses lead to allergic diseases when the IgE antibodies are produced against the apparently harmless environmental molecules, referred to as allergens. One prominent feature of IgE antibody is its property to bind via its Fc portion to specific Fcε receptors on mast cells and basophils of the same or a closely related species. The bridging of these cell-bound IgE antibodies by the complementary polyvalent allergenic molecules triggers certain intracellular events, resulting in the release of biochemical mediators. The mediators act as excitatory stimuli on smooth muscles, small blood vessels, and mucosa cells, resulting in a series of inflammatory tissue reactions(3,4).

Allergenic molecules originate from a variety of sources, including pollens, house dust mites, animal fur and danders, fungi, and foods(5,6). Grass pollens constitute one of the most important

source of allergens(7,8). The ubiquitous distribution of grass pollens renders the avoidance of contact to them almost impossible. Therefore, treatment of the pollen allergen-induced allergic diseases is particularly important. Currently, the crude extracts from grass pollens are used as reagents for diagnosis and immunotherapy of the corresponding IgE mediated allergies. One of the major drawbacks of many of these reagents is that they usually give ambiguous results in diagnosis and non-reproducible therapeutic effect(9), because of the lack of standardization of these extracts. Furthermore, since the crude extracts contain numerous components, the risk for the recipients to develop IgE antibodies and subsequent allergic responses to new components is increased. Occasionally, death by anaphylaxis is caused by using the extracts in immunotherapy(10). It is, therefore, believed that the purified allergens will improve the specificity of the diagnosis and efficiency of the immunotherapy(11,12).

The abundance of the purified allergens is expected to promote the studies towards the revelation of the mechanisms underlying the induction of IgE antibodies. Specific IgE antibody production in atopic individuals is a complicated and controlled event, which involves allergenic molecules, the adjuvant-like materials in the environment, antigen presenting cells(APC), T lymphocytes, and the antibody producing B lymphocytes which may also function as the antigen presenting cells(13-16). Currently, the development of the cellular and molecular technologies and the advances in the knowledge of allergology and clinical immunology in general make it

possible to investigate the ill-understood initiation signal(s) which triggers the synthesis of IgE antibodies.

It has been speculated that allergenic molecules, unlike the conventional antigens, have some unique structure, as a result of which interacts with particular molecules of the immune system and triggers IgE antibody production(5,17,18). However, there so far is no solid evidence that supports or overrules this speculation. In the past decade, efforts have been made to investigate the molecular structure of protein allergens from crude extracts by physico-chemical and immunochemical procedures. Little progress in understanding the nature of the allergens in terms of their molecular differences between them and the common antigens has been achieved; nevertheless progress has been made in relation to identification, isolation, and purification of allergens(5-7). Recently, gene cloning procedures have been employed in the studies of allergenic molecules, which have led to the elucidation of the complete nucleotide and amino acid sequences of allergens from diverse sources, such as *Der p* I(19) and *Der p* II(20) of dust mite, antigen-5 of hornet venom(21), *Lol p* I(22) of grass pollens, *Amb a* I(23) and *Amb a* II(24) of weed pollens, *Bet v* I(25) of tree pollens, and *Aspergillus fumigatus* allergen I(26) of fungi. The majority of these cloned allergens are clinically important, i.e., most of them were not only obtained by screening of the cDNA libraries directly with human IgE antibodies, but were also found to react with a high percentage of the individual sera from patients allergic to the corresponding crude extracts. Furthermore,

the sequence analysis of the cloned allergens revealed their relation to the other proteins and therefore defined the biological functions of these allergens (19,21,25).

Table 1. Relationships among the KBG pollen cDNA clones selected from λgt11 library

Class	Clones	Size of inserts (Kbp)*
I	KBG41	1.352
	KBG4.1	0.936
II	KBG60	1.222
	KBG1.1	0.900
	KBG8.3	0.800
	KBG10	0.500
III	KBG31	1.322
	KBG7.2	0.560
IV	KBG6.1	0.500
V	KBG5.1	0.900

*Sizes of partial cDNAs are approximations from gel electrophoresis.

In order to investigate the protein allergens at the molecular level, our group has recently employed gene cloning techniques to identify, isolate and characterize grass pollen allergens. The pollen studied in this project is from Kentucky Bluegrass, which is distributed widely in North America, Europe and Australia. Kentucky Bluegrass pollen has been shown previously by our group to contain *Poa p* I and *Poa p* IV allergens. By using the gene cloning procedures, a new group of isoallergens from this grass pollen has been identified. Initially, mRNA molecules from Kentucky Bluegrass pollen were isolated and the complementary DNAs were synthesized.

The cDNA library was then established in the λgt11 phage vector(27,28). Screening of the library with sera from patients allergic to KBG pollen allergens led to identification of several antibody-binding clones, including three full-length clones KBG60, KBG30, and KBG41, and some partial clones, summarized in Table 1(29). The sequence analysis revealed that the above three full-length clones share over 95% homology in their amino acid sequences, and that a new group of grass pollen allergens consisting of at least three isoallergens had been identified from KBG pollen. This group of newly defined allergens were designated as *Poa p* IX allergens(29,30) in accordance with the International Allergen Nomenclature system(31).

The author's study presented here mainly focus on the immunochemical characterization of a cloned *Poa p* IX allergen, rKBG60. The research was comprised primarily of the five following aspects:

(i) Synthesis of a recombinant KBG60 allergen and its overlapping fragments from Kentucky Bluegrass pollen in *E. coli*, and analysis of their allergenicity and antigenicity.

(ii) Analysis of antibody-binding epitopes of rKBG60 with synthetic peptides and their capacity to induce antibodies *in vivo*.

(iii) Allergenic and antigenic cross-reactivities of group IX grass pollen allergens.

(iv) Induction of KBG-specific IgE antibodies in mice with recombinant grass pollen allergens.

(v) Antigen- and isotype-specific immune responses to a

recombinant antigen-allergen chimeric (RAAC) protein.

A comprehensive review of our current knowledge of the mechanisms underlying generation of allergen-specific immune responses is provided below.

LITERATURE REVIEW: MOLECULAR AND CELLULAR BASIS OF THE IgE IMMUNE RESPONSES

Allergenicity is a term used to describe the property of the antigens to induce and to bind specific IgE antibodies. Production of these antibodies which is the key feature of an IgE immune response is controlled by several interacting genetic and environmental factors. Presented below is an overview of these factors which determine the allergenicity of protein allergens.

HOST GENETIC FACTORS CONTROLLING IgE PRODUCTION

The fact that only relatively a small proportion of human beings suffer from the allergic diseases despite the ubiquitous distribution of allergenic molecules in the environment suggests that the development of IgE antibody immune responses is under the control of the individual's genetic background. Indeed, genetic predisposition has been a long established feature of atopy(32). However, the genes responsible for the specific IgE antibody production to the allergens are yet to be identified.

Since MHC class II genes control specific immune responses to

the foreign antigens(33-35), the molecules are also deemed to be the genes contributing susceptibility to allergic diseases(36). Association studies between certain HLA phenotypes and allergy to specific protein allergens revealed that certain HLA class II alleles played a major role in the antibody production to the well-defined allergens. It has been demonstrated in various systems that MHC class II molecules involved in the different allergen-induced diseases differ from each other. For example, DRw52 alleles were found correlated to dust mite allergens *Der p* I and *Der f* I(37,38), DR3 alleles correlated to grass pollen allergens *Lol p* I, *Lol p* II and *Lol p* III(39-41), and DR2 alleles correlated to weed pollen allergen *Amb a* V(42,43). These results and the increasing body of literature in this field provide unequivocal evidence that MHC class II molecules are the gene products for the control of specific immune responses to the allergens. The knowledge obtained from these findings of specific interaction between MHC class II alleles and allergenic peptides may lead to the development of efficient therapeutic modalities(44).

The persistent efforts made by Marsh and his associates have led to a clear picture of the role of HLA class II molecules for the control of immune responses to protein allergens. One of the small allergenic molecules, *Amb a* V, with molecular weight of 5KDa, has been extensively studied, and these studies have led to some very important conclusions. First all, the specific immune responses to this allergen were found to be controlled by HLA-DR2/Dw2 alleles(43,45). This conclusion was drawn from the

observations that more that 95% of *Amb a* V allergic individuals possessed HLA-DR2/DRw2 genes, and the antibody responses were much higher in DR2/DRw2 positive individuals than in the gene negative individuals after immunotherapy with ragweed pollen allergens. Secondly, DR2/DRw2 alleles controlled not only IgE antibody responses but also IgG antibody responses, and the individuals with high specific IgE antibody titers had also high specific IgG antibody titers. Furthermore, it was noticed that even if most *Amb a* V allergic patients possessed the DR2/DRw2 genes, the majority of HLA-DR2/DRw2 carriers were not allergic to this allergen under normal exposure(43,45).

Further understanding of the role of MHC molecules in controlling immune responses to *Lol p* III allergen was made by the same group by studying the peptide-binding site of HLA-DR3(40,41). Southern blotting using sequence-specific oligonucleotide (SSO) slot-blot and direct sequence analysis of DNA amplified by polymerase chain reaction revealed that the antibody responsiveness was strongly associated with the sequence Glu-Tye-Ser-Thr-Ser(EYSTS). This amino acid sequence was found in DR3, DR11, and DRw6 alleles of the *Lol p* III responder individuals. Interestingly, individuals who possessed the allele with this amino acid sequence were shown to have much higher risk to develop allergy to *Lol p* III allergen than those who did not possess the alleles. These studies provide detailed information of the possible antigenic peptide binding site of the MHC molecules.

The major peptide binding regions on MHC molecules have been

shown to possess relatively broad specificities(46-48). Recent studies have shown that both class I and class II molecules possessed one peptide-binding region. Any peptide with the appropriate binding motif would fit into the binding region and compete for the site with other peptides(49-52). Thus, the defined sequence EYSTS in DR3 is most probably the binding site also for other peptides, such as peptides derived from influenza virus haemagglutinin(53,54).

Indeed, recent studies of DR2 genes between atopic and non-atopic individuals revealed that there is no difference in DR2 genes between the two groups(55). The results from the studies of DR2 and DR3 alleles in presentation of *Amb* V and *Lol p* III led the authors to conclude that MHC molecules are necessary but not sufficient to render an individual allergic to the allergens and that there might be some other gene(s) not linked to MHC contributing to the specific IgE antibody production.

The existence of other IgE controlling gene(s) has been noticed to be profoundly effective for specific IgE antibody-mediated sensitivity, often masking the effect of HLA molecule associated immune responses(56). It is generally agreed that the atopic diseases are vertically transmitted. However, the precise pattern of inheritance is still in question. Some evidence favoured that the mode of inheritance is recessive(56-58), whereas the other reports favoured the dominant mode of inheritance(59-61). The localization of the IgE antibody controlling gene(s) was not possible until the recent application of restriction fragment

length polymorphism for the analysis of genomic DNA molecules of a large atopic family(62,63). The genomic DNA from each member of the family was digested with *Taq*1 and Southern blotted with different probes. The results revealed that a 10.8Kb DNA band hybridized with a nucleotide probe specific to chromosome 11q of the atopic individuals, which was not present in the non-atopic individuals. These results indicated that a gene for atopy is localized on chromosome 11q. Interestingly, chromosome 11 has been found to carry a large number of genes for cell surface antigens, including genes involved in the immune response(64). It is now believed that specific IgE antibody production is controlled by multiple genes, one of which is MHC class II gene product which determines the specific immune response, and the other(s) may be localized on chromosome 11 or on some other chromosome determining probably the general IgE antibody responses.

REGULATION OF IgE PRODUCTION BY T LYMPHOCYTES

T cells have been recognized to play a central role in regulation of IgE antibody production. The first direct evidence that T cells were required for IgE antibody responses was obtained in the early 70's by experiments involving neonatal thymectomy, which abolished the T cells and thymocyte transfer to reconstitute the T cells, or by using a cell transfer system to re-establish the syngeneic T and B cell interaction(65-67). Thus, the helper effect of T cells for the induction and persistence of IgE immune responses was clearly demonstrated.

Okumura and Tada(68) reported that the synthesis of IgE antibodies was normally controlled by a suppressor T cell regulatory mechanism, which led to the major research interest in the field focusing on CD8+ suppressor T cells(69-72). The CD4+ T helper cells, in fact, had been overlooked. Later, considerable data from animal experiments, especially using various allergens in conjunction with different adjuvants or parasitic infection for induction of IgE antibodies, indicated that there existed some additional regulatory mechanisms for selective synthesis of antibodies of the IgE isotype. It was suggested that the synthesis of IgE antibodies was regulated by T cell secreted factors(13).

The significant breakthrough in understanding of IgE antibody regulation by T cells, however, was the discovery that in the murine system there exist two subsets of CD4+ T cells, which were functionally reciprocal in the regulation of IgE antibody production(72). These two subsets of T cells, designated as Th1 and Th2, can not currently be distinguished from their morphology and cell surface markers, but their lymphokine secreting patterns were found to be different. Whereas Th1 cells produce IFNγ, IL-2, and lymphotoxin which favour the production of the IgG2a antibody responses, Th2 cells produce IL-4, IL-5, IL-6 and IL-10, which promote the induction of IgE and IgG1 antibody responses. The existence of distinct Th1 and Th2 subsets has been recently shown also involved for humans(73).

Studies in both mouse and human systems have shown that IL-4 is required for IgE synthesis by B cells previously primed with

antigens or activated with B cell mitogen(74). IL-4 affects the IgE antibody synthesis both in vivo and in vitro, whereas the effect on IgG1 antibody synthesis has only been observed in an *in vitro* culture systems(74). Recently, the studies with IL-4 deficient mice provided the evidence that IL-4 played an essential role in both IgE and IgG1 antibody synthesis *in vivo*(75). Furthermore, it was demonstrated that at least in certain animals IL-4 deficiency is the major cause of IgE non-responsiveness(76).

The mechanism that involves signalling of the B cells to switch from production of IgM to production of IgG1 or IgE in the mouse, or to production of IgG4 or IgE in the human(77), is possibly due to a specific induction of the recombinase or an increased accessibility of the relevant regions of the Ig heavy-chain DNA(77-79). Since the finding that IL-4 induced directly germline ϵ-chain expression, the suggestions were made that, unlike switching to other isotypes, switching to IgE expression may not involve DNA rearrangement. Recent studies, however, have clearly demonstrated that IL-4 induced switch to IgE isotype involves DNA deletional rearrangement, involving the joining of S_μ to S_ϵ(80).

IL-4 is required for IgE synthesis, but it is not sufficient on its own. It was demonstrated that the optimal production of IgE antibody requires T-B cell interaction(81) and that a T cell-derived low molecular weight B-cell growth factor is necessary for IgE synthesis(82). Furthermore, in human system it was recently reported that anti-CD40 monoclonal antibody plus IL-4 is able to induce IgE antibody production via a different signalling pathway

from that of T-B cell interaction(83,84). These results suggested that different B cell activation pathways can result in IgE antibody switching in the presence of IL-4. The roles of other lymphokines secreted by Th2 have been also studied. IL-5 and IL-6 upregulate total immunoglobulin production in an isotype non-specific fashion, thus increasing IL-4 dependent IgE antibody production(85,86).

Lymphokines secreted by Th1 cells showed, however, negative regulatory effects on IgE antibody synthesis. Thus, the effect of IL-4 on IgE and IgG1 synthesis by the B cells can be inhibited by the addition of IFN-γ, which induces mainly the IgG2a immune responses(87,88). These inhibitory and enhancement effects of IFN-γ on IgE and IgG2a antibody production was first observed in *in vitro* cell culture. Injection of mice with IFN-γ also led to synthesis of IgG2a antibodies, although the doses of IFN-γ needed to induce such effect were extremely high(89). The potential application of IFN-γ in the treatment of allergic diseases was evaluated in patients with severe allergic dermatitis and the results of studies indicated that the treatment was partially successful(90).

The balance between levels of IL-4 and IFN-γ have been shown to determine the production of immunoglobulin isotypes. This knowledge has explained some experimental observations on IgE regulation made recently, such as, unlike native OA, glutaraldehyde polymerized OA does not induce substantial IgE antibody, but relatively high level of IgG2a antibodies(91). In a recent report relating to roles of Th1 and Th2 cells in the development of

cutaneous leishmaniasis suggested that IL-4 and IFN-γ might also determine to some extent the pathogenesis of diseases other than allergic disorders(92).

The role of IFN-γ in the down-regulation of IgE antibody production has been well recognized, while the role of IL-2 in the induction of IgE antibodies remains controversial. IL-2 is a growth factor for both Th1 and Th2 cells. Some reports have shown that IL-2 and IL-4 act in a synergistic manner in enhancement of IgE antibody production(93,94), whereas the others indicate that these two lymphokines inhibit each others' activities(88,95). It was demonstrated in a recent study(95) that addition of IL-2 to the B cell culture, in presence of IL-4 and LPS, inhibited IgE and IgG1 synthesis. This inhibitory effect is not dependent on IFN-γ, since the inhibition could be abolished only by anti-IL-2, but not by anti-IFN-γ. Although the mechanism(s) of inhibition is not clear, results from this study indicated that some mechanisms other than the IL-4 and IFN-γ systems are involved in the regulation of specific or non-specific IgE antibody production. One possible pathway may involve the down-regulation of the low affinity IgE receptor, CD23 and its soluble form (IgE-BF), which was found to be expressed on B cells, T cells and macrophages, and involved enhancement of IgE antibody synthesis(95-97). In another recent study it was shown by Katz and his associates showed is that Th1 cells also secreted a factor, designated as suppressive factor of allergy(SFA), consisting of two functionally and biochemically distinct protein molecules; one of these molecules with M.W. of

17KDa decreases the interaction between IgE antibody and CD23 on B cells, whereas the second protein with M.W. of 30KDa suppresses IgE antibody synthesis(98).

Even though several mechanisms, perhaps including also CD8+ suppressive T cells, appear to be involved in regulation of IgE antibody production, Th1 and Th2 cells are believed to play major roles in the controlling IgE antibody synthesis. Several studies have indicated that Th1 and Th2 might originate from the same parent Th0 cells which have the potential to synthesize both IL-4 and IFN-γ(99-101). If this interpretation proves correct, there remains question as: "What is the signal(s) triggering the differentiation of Th0 into Th1 and Th2 cells?"

MOLECULAR BASIS FAVOURING CLASS SWITCH TO IgE ANTIBODIES

Class switching occurs by molecular recombination between switch (S) regions located upstream of each heavy chain constant region gene accompanied by intramolecular deletion of the DNA segment between the recombined S regions(102,103). In the mouse, there are eight immunoglobulins isotypes, each encoded by a distinct immunoglobulin C_H gene. The order of genes on chromosome 12 is: 5' -μ, δ, γ3, γ1, γ2b, γ2a, ϵ, and α -3'. Although it is well recognized that the switching to these different immunoglobulin isotypes is from the parent IgM immunoglobulin, the mechanism underlying the specific switch of a given B cell to the production of a certain isotype is not clear. Recent studies revealed that certain factors may be responsible for the isotype

switching, viz., (i) lipopolysaccharide(LPS), stimulating B cells to express IgG3 and IgG2b antibodies; (ii) transforming growth factor β, inducing IgA antibody expression; (iii) interferon-γ, enhancing the switch to IgG2a antibody; (iv) interleukin-4, responsible for the switch to IgG1 and IgE antibodies(73,87-89).

The mechanisms of the effect of IL-4 on isotype switching to Cγ1 and Cϵ have been linked to the previous finding that IL-4 induces a trans-acting factor, which binds to the cis-element upstream of the class II Aα^K gene and activates the gene transcription(104). In that study, the DNA footprinting technique was employed. After incubation with nuclear extract from the IL-4 induced spleen B cells, the DNA fragments generated by restriction digestion were digested with DNAase I, which led to the identification of two pieces of DNA fragments, named as BRE-1 and BRE-2, protected by the trans-acting factor from further digestion by DNAase I. Further sequence analysis revealed that the two fragments shared an identical motif, 5'-ATG-TGC-T--AAG-3', although the remaining sequences of these two fragments are different. Inhibition assays performed with the synthetic oligonucleotides demonstrated that BRE-1 and BRE-2 can reciprocally inhibit each other's binding to the IL-4 induced trans-acting factor; thus, this evidence demonstrates that BRE-1 and BRE-2 recognize the same protein. Recent studies demonstrated that there exist highly homologous nucleotide sequences to BRE-1 or BRE-2 in the sequences upstream from the initiation codons of the germ-line Cϵ and Cγ1 transcripts(105,106). Deletional analysis of the sequences led to

the same conclusion as in the study of $A\alpha^K$ gene that the BRE-1 homologous sequence is the binding site of IL-4 induced trans-acting factor(106).

Germ-line transcription of a particular immunoglobulin gene is believed to have a regulatory function in the corresponding isotype switching, although the mechanisms underlying the increase of the accessibility of the particular S region by its preceding germ-line transcript are unclear. In the Cε system, it seems that the cis-element binding to the IL-4 induced trans-acting factor functions only at the germ-line transcription level and may not have a direct effect on the productive isotype switching(79,80,105). The germ-line transcripts generated thereafter somehow influence the accessibility of the S_ϵ region. Generation of the productive ε transcripts requires an additional signal besides IL-4(83, 84). Previous extensive studies have shown that the additional signal(s) for IgE antibody induction can be provided by the activated T cells or their membranes, EB virus, LPS, and anti-CD40 monoclonal antibodies. These studies have been extended to the other T and B cell surface molecules. The results demonstrate that monoclonal antibodies to CD2, CD19, CD21, CD40, and CD45 augment IgE production, whereas antibodies to CD3, CD4, DP, DQ, and DR inhibit IgE production, when the cells are cultured in the presence of rIL-4(107).

The above studies suggest that the Cε germ-line transcript induced by IL-4 is essential for productive Cε transcription, whereas the additional signals can be provided by variety of

molecules. Studies of one relatively well characterized B cell surface molecule, CD40, indicated that different pathways were involved in the B cell activation lead to IgE switching in the presence of IL-4. The evidence supporting this argument is from the comparison of signals provided by the activated T cells and anti-CD40 monoclonal antibodies(84). The differences in the signal transduction pathways between T cell direct contact and anti-CD40 stimulation were demonstrated by the analysis of their abilities to induce Cε germ-line transcripts and inhibition of their effects on IgE synthesis with IFN-γ and IFN-α. These different additional signals, such as direct contact of T and B cells and anti-CD40, besides the IL-4 leading to IgE synthesis are indicated to play a similar role in Cε isotype switching once the germ-line Cε transcripts were induced by IL-4. However, this does not mean that the additional signals are not critical in IgE synthesis. Indeed, recent results obtained by using a nested PCR strategy clearly demonstrated that isotype switching from μ to ε occurs only in the presence of the additional stimulator rather than only in the presence of interleukin-4(80,108).

From the above studies, it can be concluded that Cε switching is controlled interleukin-4, which induce the germ-line transcripts preceding to the productive Cε transcription. For the productive Cε transcription, at least one additional signal is needed at the stage of recombination. This signal may be provided by cell-cell interaction, polyclonal mitogen such as LPS, and other factors interacting with variety of the cell surface molecules(73, 87-89,

107). Elucidation of the additional signal transduction pathways provided by the different molecular interactions is essential for the complete understanding of IgE antibody production.

ROLE OF ADJUVANT-LIKE MATERIALS IN THE INDUCTION OF IgE ANTIBODIES

In experimental animal models, it is well recognized that adjuvant is essential for the production of antigen or hapten specific IgE antibodies(3,17). The adjuvant may influence either T cells or B cells, thus promoting the differentiation of T cells to various functional subsets or of B cells to secrete different isotype antibodies. It is generally accepted that some adjuvants, such as complete Freund's adjuvant, promote Th1 cell proliferation thereby inhibiting Th2 cell activity and IgE antibody production, but other adjuvants, such as alum, have the opposite effect. Recently, studies of some B cell stimulators demonstrated that they are also important in determinating isotype of the antibody being produced. Unlike LPS which activates all different isotype-committed B cells and preferentially induces IgG3 and IgG2b expression(109), anti-IgD, when conjugated to dextran (anti-immunoglobulin D-dextran, AIDD), induced no IgE secreting cells although induced IgG1 secreting cells from the resting B cells with or without the presence of IL-4(110). Interestingly, in the same study it was demonstrated that anti-IgD, when conjugated to sepharose, induced IgE antibody production in the presence of IL-4. Furthermore, if IFN-γ was added to AIDD-stimulated culture, IgG2a antibody was induced. These results strongly suggest that different

pathways involving various B cell membrane receptors and signal transduction mechanisms are involved in B cell activation, which may lead to the differentiation of the B cells into antibody-secreting cells of a particular isotype.

It was also noticed that immunization with a given antigen along with an infective parasite, such as *N. brasiliensis*, enhanced the antigen-specific IgE antibody production(111). This effect is believed to be due to some substance(s) in the parasites which preferentially stimulates Th2 type immune responses and promotes at the same time non-specifically the induction of IgE antibody to the co-injected antigen. This hypothesis was supported by recent studies of two immuno-active reagents, cyclosporin A and ricin(112,113). Analysis of the effect of cyclosporin A, an immunosuppressive drug, on immune responses revealed that this drug is in fact an immunomodulator which generally inhibits immune responses, but preferentially potentiates IgE antibody responses regardless of Ag specificities(112). Similarly, the effect of ricin, a castor bean toxin, has been also reported to potentiate IgE antibody production(113). Antigens injected with ricin induce substantially higher levels of IgE antibodies than without using the toxin. Although the mechanism(s) underlying the preferential induction of IgE antibodies by cyclosporin A and ricin is not clear at present, the results obtained suggested that both cyclosporin A and ricin inhibit IgE suppressor cells, thereby leading to the hyper-IgE production to the co-injected antigens(112-114).

Certain environmental factors play an adjuvant-like role in

the determination of IgE antibody production to common allergens under natural conditions. For instance, industrial dust has been found to contain substances which induce IgE antibodies. Among these substances some can induce IgE antibody directly when adsorbed to the protein carrier, and the others potentiate the immune system to produce IgE antibodies to the protein allergens(115-119). The well characterized materials in the former category are the low molecular weight chemicals, such as acid anhydrides and isocyanates. These chemicals can bind to proteins and directly induce specific IgE antibody production(115,116). The other kinds of industrial dust, such as aluminium silicate and particulates of diesel exhaust, were found to have an adjuvant-like effect for IgE antibody production in animal models(117,118). Although these results were obtained under the experimental conditions, it is not unlikely that the dust may stick to the common aero-allergens and promote specific IgE antibody production. Furthermore, tobacco smoke was recently reported to impart an adjuvant effect on the proteins and to potentiate IgE antibody production. This effect was found in a mode of the non-specific stimulation or of the enhancement of antigen-specific antibody production(119).

The existence of a variety of adjuvant-like substances in the environment, some of them confirmed in experimental animals, reflects the possibility of diverse mechanisms for the activation of T and B cells. Recent progress in the knowledge of T and B cell surface markers, i.e., clusters of differentiation(CD) antigens,

has led to the discovery of at least 20 of these on T cells and 10 of these on B cells, which are involved in signal transduction and activation of the cells(120).

ROLE OF ANTIGEN PRESENTING CELLS IN IgE PRODUCTION

As stated earlier, the specific IgE antibody production is controlled by MHC class II molecules, which are expressed by certain types of cells, including the macrophage, dendritic cell, B cell and Langerhans cell. It is clear that these different cell types in the same individual express the same class II molecules. However, the observation that different forms of antigens resulted in activation of different T cell subsets(121,122), led to the consideration of the essential role of APC in T cell differentiation. Recently, direct investigation of different types of APCs in antigen presentation confirmed that these cells influence the differentiation of T cells and antibody production by B cells(124-128).

It has been demonstrated that the allergic reaction is a major cause of dermatitis(123), and consequently epidermal Langerhans cells were studied for their roles in specific IgE antibody production. It was thus shown in *in vitro* culture system that hapten-modified Langerhans cells preferentially induce CD4+ T cells, in either autoreactive or antigen specific mode(124). It was found that Langerhans cells can stimulate T cells, leading to synthesis and secretion of IL-4. When these *in vitro* activated T cells were cultured with the syngeneic resting B cells in the

presence of the antigen, production of IgE antibodies specific to the primed hapten was observed. Furthermore, by using ultraviolet B radiation it was shown that the capacity of the Langerhans cells to present certain antigen to Th2 cells was higher than that to Th1 cells(125,126). In these studies, the Langerhans cells were found normally to be able to present KLH to both Th1 and Th2 cells. However, after the low dose radiation, the Langerhans cells lost their ability to stimulate Th1 cells, without affecting at all the stimulation effect on Th2 cells.

More recent studies focused on two another types of antigen presenting cells, macrophage and B cell, which showed reciprocal function in activation of Th1 and Th2 cells(127,128). It is generally agreed that large antigen particles, such as viruses, bacteria and foreign cells, are processed and presented by macrophages, whereas soluble antigens are processed and presented by the more efficient B cells. The first evidence suggesting that macrophages may only be involved in presenting antigen to Th1 cells was obtained by studying hepatic accessory cells(127). The results from this study demonstrated that the macrophages from murine liver have the ability to stimulate cell proliferation of Th1 type but not Th2 type. The same group further analyzed splenic macrophages in comparison to splenic B cells with respect to their capacity for antigen presentation. The results showed that the spleen cells could present ovalbumin (OA) to and stimulate both Th1 and Th2 cells, whereas the fractionated adherent and B cells activated only Th1 and Th2 cells, respectively (128).

The basis of the difference between macrophages and B cells in terms of their capacity to activate distinct subsets of T cells is as yet unknown. However, several indirectly related studies may provide clues for the understanding of these mechanisms. First, it was found that the requirements for expression of Ia molecules on the surfaces of macrophages and B cells were different. While B cells require IL-4 to increase the surface expression of Ia molecules(129), the expression of Ia molecules on macrophages was influenced by IFN-γ(130). Therefore, when resting B cells or macrophages were used as APCs, their antigen presenting activities appeared to be largely influenced by the co-cultured T cell types, i.e., Th1 cells mainly activate macrophages and Th2 cells primarily activate B cells(128). Secondly, it has been demonstrated that activation of T cells requires at least two signals; one is from the peptide-MHC and TCR interaction, and the other involves two surface molecules, such as CD28 and B7 molecules on T cell and B cell, respectively(131).

Hence, it is reasonable to assume that B cells and macrophages may express different surface molecules, which are involved in the signal transduction and lead to different T cell responses. The assumption that different molecules on diverse cells may play a role in immune responses is supported by the observation that even B cells themselves do not represent a homogenous population. Recent studies of IgE positive B cells demonstrated that this population carries the Thy-1 marker, which is absent or present at very low frequency on the other Ig isotype B cells(132). Moreover, the

proteinases and proteinase inhibitors within macrophages and B cells are apparently different, which may contribute to the different epitope presentation by the different cells(133). One well-documented example is dehydroepiandrosterone(DHEA) sulfatase, which was found present mainly in macrophages of the lymphoid organs. This enzyme was believed to create a DHEA-rich microenvironment, and DHEA was found to enhance the differentiation of T precursor cells to Th1 cells(134,135).

It is obvious that APCs play an important role in determination of T cell differentiation and B cell isotype antibody production. As stated above, multiple factors may be involved in antigen presentation by APCs. Research on cell surface markers and their function and proteinase activities in the different APCs is expected to shed light on the mechanisms involving the effect of APC on IgE antibody production.

ROLE OF ALLERGEN MOLECULES IN DETERMINATION OF IgE PRODUCTION

As previously reported, Th1 cells specific to allergens were found in non-atopic individuals, i.e., cells which do not provide help to autologous B cells to produce IgE antibodies(136,137). Moreover, the results from the analysis of the sequence homologies among the cloned allergens are somehow discouraging so far in terms of revelation of any common properties that may lead to stimulation of IgE antibody production. However, a few recent observations presented below indicate that in the same human individual, or experimental animals with the same genetic background, the antigens

may determine the direction of isotype immune responses.

The first indication is from the studies of T cells from the atopic patients. The subsets of T cells, Th1 and Th2, were used as markers to examine the effect of different protein antigens on T cell activation and proliferation. The results obtained from different laboratories are strikingly similar(138-141), i.e., the antigen-specific T cells from the same individual were found to have two compartments, one is Th1 cells with specificities mainly to bacterial antigens, including tetanus toxoid and antigens from *Candida albicans*, and the other is Th2 cells with specificities mainly to common allergens, including dust mite and grass pollen allergens. These results strongly suggest that under natural exposure, common antigens activate the T cells to differentiate to Th1 type cells, while the allergens preferentially stimulate the T cells to differentiate to Th2 type cells.

Moreover, indirect evidence suggests that the dose and route of exposure and adjuvant-like materials may not play an essential role under natural exposure, since the antigens in the crude extract from the same source have been reported to induce different types of immune responses(142-145).

One study of fungal infection with *Tichophyton tonsurans*, from which most antigens induce delayed type hypersensitivity(DTH), revealed that a 30KDa protein antigen induced only immediate hypersensitivity but not DTH one(142). More convincing results are from the studies of different antigens of *Leishmania major* that induced either Th1 and Th2-like responses when used separately to

immunize experimental animals against challenge of the pathogens(143). Similar phenomena were also observed by our group and others in the studies of grass pollen aqueous extracts, i.e., only some of the antigenic components in the grass pollen extracts were recognized by human IgE antibodies(144,145). A these observations taken together reveal that proteins from the same sources possess different capacities of induction of IgE antibodies. Moreover, since the protiens are from the same sources and both allergenic and non-allergenic proteins exist in different amount in the exact, the differences of abilities of these protein antigens to induce IgE antibodies indicate also that dose and route of exposure, and possible adjuvant-like materials associated with allergens may not be sufficient to explain why certain proteins are allergens.

More impressive results were obtained recently by use of experimental animals in investigation of the immunogenicity of two unrelated peptides. One is a natural 26 amino acid peptide, melittin from bee venom, and the other is a synthetic peptide at position 12-26 derived from bacteriophage λ cI repressor protein(146,147). There are two striking similarities between these studies, which are (i) both peptides induced specific IgE antibodies irrespective of the adjuvant used and (ii) changing amino acid residues altered the capacity for their IgE antibody induction. In these studies, it was demonstrated that both complete Freund's adjuvant and $Al(OH)_3$ had adjuvant effects on the specific IgE induction to the peptides. While a single amino acid change

(addition of an amino acid Tyrosine at position 27) of the λ cI peptide resulted in a total loss of its ability to induce IgE antibody(146), studies of melittin showed that a peptide of at least 24 amino acids in length and present at hydrophillic C-terminal region preferably with two to four cationic groups was essential for induction of IgE antibodies(148).

Taken together these observations indicate the possible role of allergen molecules in determination of IgE production in the atopic individuals. Detailed analysis of the molecular structures, including T and B cell epitopes, tertiary structure and possible motifs, of allergen molecules may lead to some more convincing results.

B- AND T- CELL EPITOPES OF ALLERGENS

A protein allergen, like other protein antigens, is comprised of (i) B cell epitopes which are regions of antigens specifically recognized by the corresponding B cells and their secreted antibodies, and (ii) T cell epitopes which are associated with MHC molecules in the form of complexes, i.e., T cells recognize these complexes.

B cell epitopes of allergens

B cell epitopes of protein antigens have attracted general interest in the field of immunology, since that detailed analysis of the structures of the epitopes is expected to help understand the molecular basis of protein antigenicity with respect to: (i)

the mechanism of antigen-antibody interaction, (ii) the potential usage of synthetic peptides as vaccines and (iii) the ability of various algorithms to predict the location of antigenic sites in proteins based solely on amino acid sequence data(149).

In addition to the above general interest, definition of allergenic epitopes enables one to examine the possibilities involving immunotherapy. An example of the application of B cell epitopes is based on the well-established mechanism of allergic reaction, i.e., bridging of two IgE molecules on mast cells or basophils by multivalent allergen or divalent anti-IgE is required for triggering histamine release(150,151). Indeed, a peptide synthesized from a cod fish allergen was shown to block but not elicit allergic reactions(152). Thus, the well-defined and purified monovalent allergenic peptides are expected to abrogate allergic reactions by interfering with the primary interaction between the native allergen and IgE antibodies.

The other attempt is to induce specific suppression of IgE antibody production to the allergenic epitopes. This consideration is based primarily on the observations that, in various hapten-carrier systems, immunization of carrier-primed mice with a new epitope coupled to the priming carrier induces suppression for high affinity IgG antibody response to the newly introduced epitope without interfering with the response to determinants on the carrier molecules(153,154). Indeed, a recent report demonstrated that this so-called epitope-specific regulation is because the hapten-specific memory B cells have an intrinsic defect that

prevents differentiation of the cells into active IgG antibody secreting cells(155). Furthermore, the allergenic epitopes may linked to antigenic molecules or may be directly modified as reported previously(156) to provide a potentially useful modality to modulate IgE antibody synthesis.

B cell epitopes were found to be either sequential or conformational(157). Recent studies, however, on the crystal structure of antibody and antigen complexes have provided an updated view that most, if not all, of the epitopes are conformational, containing more or less dominant sequential elements(158). By comparison of native and denatured allergens, it was demonstrated that IgE binding epitopes, like the other antigenic epitopes, are also different in their conformational or sequential nature(159-162). In these studies, it was also indicated that carbohydrate groups contribute to IgE binding for only some but not all allergens in a mode of either being direct binding sites or just keeping the conformation of the proteins.

To localize the epitopes on protein antigens, different approaches have been employed, with diverse results in terms of size, composition and location of epitopes. The approaches that have been used for epitope mapping of protein antigens were summarized by Regenmontel(149) and presented in Table 2 with modification.

It should be noted that in Table 2 only method 1 can be used to visualize the spatial arrangement of the structure of the epitope involved in the antibody binding. Instead, the other

methods are virtually functional analysis of the epitopes. A notable disadvantage of the method of crystallography is that it provides no information on essential amino acids in the epitopes, whereas the other methods are useful to pin-point the critical residues.

Table 2. Methods used to localize epitopes in protein antigens

Method	Type of epitope recognized	Average number of residues identified in epitopes
1. X-ray crystallography of antigen-Fab complexes	discontinuous epitope reacted with homologous antibody	15
2. Study of cross-reactive binding of peptide fragments with anti-protein antibodies	Continuous epitopes cross-reacting with heterologous antibody	3-8
3. Study of cross-reactive binding of protein with anti-peptide antibodies	Continuous epitopes cross-reacting with heterologous antibody	3-8
4. Determination of critical residues in peptide by systemic replacement with other amino acids	Continuous, cross-reacting epitope containing critical residues interspersed with irrelevant residues	3-5
5. Study of cross-reactivity between homologous proteins or point mutants	Discontinuous epitopes	1-3

Because of the paucity of purified allergenic molecules and difficulties in obtaining monoclonal IgE antibodies for epitope-analysis using techniques of crystallography, the crystal structure

of IgE-binding epitopes is not available. Thus, there is no report of discontinuous IgE epitopes yet. However, by using the other means, continuous IgE binding epitopes were defined in detail for a few allergens.

The first allergen to be characterized with respect to epitope structure was allergen M (*Gad c* I), which is a 12KDa calcium binding parvalbumin of white muscle tissue from cod fish. Employing techniques of trypsin cleavage of the native protein or synthetic peptides, Elsayed et al(163) identified five IgE binding distinct sequences of the 113 amino acid long *Gad c* I. These sequences located at positions 13-32, 33-44, 49-64, 65-74 and 88-96, were found to be responsible for the immunological reactivities of the native protein. It is noteworthy that these five peptides do not represent the full profile of IgE binding epitopes of the allergen, since the authors showed that peptides 13-32 and 49-64 were at least divalent in nature. The peptide 49-64 encompassed two repetitive sequences D-E-D-K and D-E-L-K separated by a hexapeptide spacer. Interestingly, this unique repetitive structure was found to be cross-reactive with birch pollen allergens(164).

Another well-characterized allergen, the non cell-bound haemoglobin of chironomid larvae (*Chi t I*), is from the insect family Chironomidae(165). *Chi t I* was found to be composed of 12 homologous components. Epitope mapping of a component *T III* (136 amino acid in length) using trypsin-digested peptides revealed that three of eleven peptides, at positions 1-15, 91-101 and 110-135, were able to react with human IgE antibodies. These results are

slightly different from the previous quoted report of the component *T IV*(166), since a peptide at position 32-90 from *T IV* was found also reactive with human IgE antibodies. This difference may be due to the lengths of peptides from the digestion of component *T III*. Within the region of amino acid residues 32-90, six peptides were generated from *T III* and none of them was longer than 22 amino acids(165).

A comprehensive synthetic approach for delineation of continuous epitopes was introduced by Atassi and Atassi(167,168). This approach, which used series of overlapping synthetic peptides, greatly facilitated the identification of IgE binding epitopes of *Amb a* III, an allergen from ragweed pollen. The authors synthesized a series of 15 residue-peptides, with five amino acid overlapping one another, spanning the entire 101 amino acid residues of the protein. Epitope-mapping lead to a result that five peptides, at positions 1-15, 21-35, 31-45, 51-65 and 71-85, were found to be able to bind to IgE antibodies(168).

Currently, another low cost epitope mapping method developed by Geysen et al(169) based on solid phase peptide synthesis and ELISA assays is widely used for analysis of protein antigenic sites. The method has been successfully employed to define variety of antigenic epitopes(169); however, so far, it appears that the results are not satisfactory from the limited applications of this method for identification of IgE binding epitopes(170,171). The reasons for these unsatisfactory results may be twofold: the purity, quantity and lengths of the peptides on the rods and the

low titers of IgE antibodies in human sera.

Recently, recombinant DNA techniques were also employed for mapping IgE binding epitopes. An allergen, *Der p* I, from dust mite was cloned and sequenced(19). With the recombinant peptides obtained from either a random fragment library or restriction digestion, Thomas and co-workers were able to define the IgE binding sites of this allergen(172). Out of 16 overlapping recombinant peptides spanning the entire molecule of 220 amino acids, at least five regions, comprising residues 1-56, 53-99, 98-140, 166-194 and 188-222, were found to be reactive with human IgE antibodies. An advantage of various lengths of the 16 overlapping peptides, ranging from 114 residues to 14 residues, led to an important conclusion that IgE binding peptides of *Der p* I were at least 30 amino acid residues long.

Until recently, the above four allergens have been investigated in detail for their profiles of allergenic determinants. Defined IgE-binding peptides, but not systematic analysis, from various sources such as ovalbumin and pollens have been also reported(164). These studies have led to the two main conclusions, i.e., (i) although it was observed that polar or charged amino acids were often in the epitopes(163-166), there exist virtually no consensus sequences among these IgE-binding peptides, and (ii) all the above IgE-binding peptides were found also to be reactive with IgG antibodies(163-172). Indeed, previous results from application of monoclonal antibodies for inhibition of human IgE to bind to the allergens indicated the same conclusion

that IgG and IgE recognize the same or adjacent epitopes(173,174).

Roles of T cell epitopes in activation of different subsets of T cells

By contrast to the majority of B cell epitopes, T cell epitopes are sequential in nature composed of mostly 9 amino acids for $CD8^+$ T cells and relatively diverse lengths for $CD4^+$ T cells(47-52). Since T cells play an essential role in regulation of IgE antibody production, T cell epitopes have recently received more attention than the B cell epitopes of the allergens.

Again, because of paucity of information of sequences of allergens, T cell epitopes were only defined from a limited number of allergens. One well-characterized allergen is *Amb a* III. Attassi et al defined T cell stimulatory peptides at positions, in decreasing levels of responses, 51-65, 1-15, 11-25, 81-95, 71-85 and 91-101 by using ten 15 residue overlapping peptides(175). Three of these peptides were found in this study to be coincident with B cell epitopes. A later strategy, induction of antibody production *in vivo* directly with synthetic peptides, was used to examine all the antibody-binding peptides. The results showed that all the five antibody-binding peptides possessed also T cell epitopes therefore the antibodies which recognized both peptides and native protein were induced(167).

Lol p I allergen was also analyzed its T cell epitopes with peptides synthesized based on the sequences obtained from deduction of nucleotide sequences of a cDNA clone(22). Twenty-three 20

residue peptides representing the entire 240 amino acid protein, with 10 amino acid overlapping, were examined with four human T cell clones specific to *Lol p* I allergen. Interestingly, it was found that all four clones selected from *Lol p* I-stimulated PBL recognized peptide number 20 at position 191-210, indicating this peptide represented an immunodominant T cell epitope of the allergen.

The role of T cell epitopes of allergens in activation of different subsets of T cells is unknown. However, some indirect evidence from studies of TCR in cell differentiation and T cell epitopes from proteins other than allergens suggested that TCRs and their ligands, T cell epitopes, might be involved in the T cell subset differentiation(176-179).

As stated earlier, Th1 and Th2 cells may originate from the Th0 cells depending on the differential stimulation(99-101). One recent report suggested that T cell differentiation from Th0 cells to either Th1 or Th2 cells does have a relation to the TCR complex(176). In this report, the authors investigated the short-term cultured murine CD4+ T cells. The results showed that if the cells activated by staphylococcal enterotoxin were cultured with IL-2 and restimulated with the superantigen, the cells expressed Th1 phenotype at day 11. However, addition of anti-CD3 monoclonal antibody to the culture led to the expression of Th2 phenotype during the same time. This observation was confirmed by the application of anti-Vβ8 instead of anti-CD3 monoclonal antibody in the same culture. The results showed that Vβ8+ but not Vβ8- T cells

were induced to differentiate into the Th2 phenotype cells. The strong argument from this study is that the TCR, the receptor of T cell epitope and MHC molecule, is critical for Th2 cell differentiation.

T cell epitopes, the natural ligands of TCR, seem also to have effects on the cell differentiation. Previously, it was shown from variety of studies that different antigens in the same crude extract differed in their antigenicity and allergenicity(142-145). At the epitope level, recent studies of a 19KDa bacterial protein from *Mycobacterium tuberculosis* showed that different regions of the antigen had preferential effects on activation of different subsets of T cells(177). In this study, the overlapping synthetic peptides spanning the antigen of 159 amino acid residues were used to define the T cell epitopes. Five peptides at positions 1-20, 61-80, 76-95, 136-155 and 145-159 were found to be able to stimulate the antigen-specific T cells in proliferation assays, therefore, the T cell epitopes of this antigen were defined. While peptides 76-95 and 136-155 were found relatively weak in induction of both Th1- and Th2-like cells, peptides 1-20 and 61-80 induced preferentially Th2 type responses whereas peptide 145-159 induced dominant Th1 type response. Together it was suggested that the small molecular structure of T cell epitopes play a role in activation of different subsets of T cells.

Detailed structures of Th1 versus Th2 epitopes were analyzed by Allen and his associates(178,179). The report indicated that substitution of a single amino acid of a T cell epitope resulted in

loss of its ability to stimulate T cell proliferation, while retaining its ability to induce the cells to secrete IL-4(178). It was concluded from this observation that T cell proliferation and cytokine secretion are two different events and the amino acid residues in the T cell epitope are critical to these events. Another study from the same group indicated that there may exist fine differences between Th1 and Th2 epitopes(179). In this study a 12 residue peptide at position 64-76 derived from murine heamoglobin was employed to generate a panel of specific Th1 and Th2 clones by immunization of mice with the peptide in complete Freund's adjuvant. With the serial peptide analogues the authors demonstrated that both Th1 and Th2 cell clones require the same essential amino acid residues. However, some differences in fine specificity of the Th1 and Th2 cell clones was noticed, such as phenylalanine at position 71 is critical for the Th1 cell recognition, whereas substitution of this amino acid has no effect on the Th2 cell stimulation. Thus, although the peptide could induce both Th1 and Th2 subsets and the cells of both subsets shared the gross specificities, the fine differences in their specificities remain an interesting issue for further investigation.

The only allergen, of which T cell epitopes have been functionally analyzed, is the bee venom melittin. Previous studies demonstrated that its C-terminal (amino acid residues 20-26) with cationic residues were essential for specific IgE and IgG1 antibody production(148), indicating the major T cell epitope(s) in this

region was critical for Th2 cell activation. Further studies of this molecule led to the finding that its middle region also possessed a T cell epitope at position 11-19(180). This peptide was an α-helical amphiphilic segment of the molecule and was able to induce Th2 cells in mice. Substitution of the amino acids of the peptide and analysis of its secondary structure indicated that the primary structure of this peptide was more important than its secondary structure for its ability to induce Th2 cells(180).

In essence, bulk of evidence to date suggest that the B-cell epitopes of allergens are able to react with both IgE and IgG antibodies, although some epitopes reacting with IgE but not IgG and vice versa have been reported. The roles of T cell epitopes, if any, on T cell differentiation from Th0 cells to Th1 or Th2 subsets, thereby influencing immunoglobulin class switching are not clear as yet. Although the results discussed above indicate the different effects of T cell epitopes on activation of T cell subsets, more detailed analysis of a variety of such epitopes, in particular the ones from allergens, are required for determination of their roles.

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CHAPTER II

SYNTHESIS AND IMMUNOLOGICAL CHARACTERIZATION OF A RECOMBI NANT KBG60 ALLERGEN AND ITS FRAGMENTS

ABSTRACT

A gene cloning and expression procedure was employed to synthesize a large amount of a recombinant *Poa p* IX allergen, rKBG60, and its four overlapping fragments, rKBG60.1, rKBG8.3, rKBG10 and rKBG60.2, in *E. coli*. The cDNA inserts coding for the allergen and the fragments were introduced into the plasmid vector pWR590.1. High-level expressions of these cDNA clones were obtained in strain JM105, with the expression levels ranging from 15% to 40% of the total cell proteins. All the recombinant allergenic polypeptides were synthesized in a form of a fusion protein, associated with a truncated β-galactosidase, and found to be reactive to IgE antibodies. Thus, it was demonstrated by ELISA using human IgE and murine IgG antibodies that the epitopes were on all the fragments. Furthermore, inhibition analysis of the antibody binding to the rKBG60 with two fragments, rKBG60.1 and rKBG8.3 representing N- and C-terminal regions of the full-length of the allergen respectively, revealed that the majority of human IgE epitopes were located on a C-terminal region. Moreover, three natural allergens in KBG pollen extract, with molecular masses of 28KDa, 32KDa and 34KDa respectively, corresponding to the recombinant allergens were identified by western blotting using antisera raised against the rKBG8.3, which represented the conserved region of the *Poa p* IX allergens.

INTRODUCTION

The development of more efficient immunotherapy has been hampered by the paucity of defined and purified protein allergens. In the case of immunotherapy of grass pollen allergies, currently crude exacts of the appropriate pollens are used. Obviously, these extracts contain a multiplicity of constituents, most of which may bear no relation to the few allergenic components that are present in minute amounts and are responsible for a given patients' hypersensitivity. Hence, the use of the extracts results often in inconsistency of therapeutic effect, even occasionally leading to harmful side effects(1-3). The availability of the purified, standardized allergens seems to have considerably improved the efficacy of immunotherapy and specific discriminating diagnostic assays(3-5).

Moreover, the availability of the purified allergens is also essential for studies of immunochemical properties of the allergens. These studies are expected to elaborate the structural basis of allergenicity. Another equally important question, i.e., whether the antigenic and allergenic sites on the allergens are the same remained unsolved. Most reports indicated that IgG and IgE antibodies recognize the same sites(7-11), although some reports indicated that IgG and IgE binding sites on cod Gad c I allergen may be distinct(6). Elucidation of the differences between IgG and IgE antibody induction are fundamental, therefore, for understanding of the mechanisms underlying the induction of

hypersensitivity.

Conventionally, isolation and purification of the allergens from the crude extract have been achieved by employing physicochemical methods and reverse immunosorbents(12,13). Although these methods have proven useful, one of the major drawbacks of these labour-intensive purification procedures is the low yield of the pure allergens. Thus, it is long overdue to apply the new recombinant DNA (rDNA) techniques to isolate and purify the allergens. Recently, the rDNA techniques have been employed for the studies of variety of clinically important allergens from dust mite, hornets and tree pollens, which led to cloning, sequencing, and expression of these allergens(14-16). However, studies with grass pollens were limited.

Contemporary to the above studies, we constructed a cDNA library in λgt11 phage vector with the mRNA isolated from Kentucky bluegrass pollen(17). Upon screening of the library with sera from the pollen allergic patients, it was apparent that several clones had been isolated(18). Sequence analysis of these cDNA clones by Andre Silvanovitch (Fig.1) revealed that they encoded a new group of proteins, which was designated as *Poa p* IX allergens(19). Although the vector λgt11 we used is very efficient in cloning, the difficulty of its manipulation and relatively low level of expression of the inserted genes make it unsuitable for the purpose of obtaining a bulk of purified recombinant allergens. In the present study, the cDNA clone coding for one of the *Poa p* IX allergens, rKBG60, has been introduced into a plasmid expression

vector, pWR590, to achieve high-level expression. The overlapping fragments of rKBG60 were also generated using the same vector, which were analyzed for their antibody-binding activities.

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41   -23 mavhqytvalflavalvagpaas
60   -22 ...qk.......v. ..v.....
31   -28 .dkangaykta.ka.sava..ekfpvfqATFDKNLKEGLSGPDAVGFAKKLDAFIQTSYL

41     1                         YAADVGYGAPATLATPATPAAPAAGYTPAAPAGAAPKA
60     1                         ....LS......      ......A.............
31    33 STKAAEPKEKFDLFVLSLTEVLRFMAGAVK..PASKF..K..PKV.A.............

41    39 TTDEQKLIEKINAGFKAAVAAAAGVPAVDKYKTFVATFGTASNKAFAEALSTEPKGAAAA
60    33 .............V.........G....AN..........A..................VD
31    93 .............V..............AS..........A..................V.

41    99 SSNAVLTSKLDAAYKLAYKSAEGATPEAKYDAYVATLSEALRIIAGTLEVHAVKPAGEEV
60    93 ....A............................D...................G....A...
31   153 ..................................................G....A...

41   159 KAIPAGELQVIDKVDAAFKVAATAANAAPANDKFTVFEAAFNDAIKASTGGAYQSYKFIP
60   153 ..T.....................................................
31   213 .....................................................

41   219 ALEAAVKQSYAATVATAPAVKYTVFETALKKAITAMSQAQKAAKPAAAVTATATSAVGAA
60   213 ..............................................A.G.........
31   273 ..............................................G.........

41   279 TGAVGAATGAATAAAGGYKTGAATPTAGGYKV
60   254 .                   .....AA......
31   333 .                   .....AA......
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Fig.1. Translated sequences of KBG clones 41, 60 and 31. Sequences are aligned and gaps aligned to show maximum similarity between the clones. Dots represent homology with clone KBG41. Putative signal peptides are in *lowercase characters*. Numerical values at the start of each line refer to the clone and the number of amino acid residues from putative cleavage site of the signal peptide.

MATERIALS AND METHODS

Preparation of cDNA clones, KBG60, KBG8.3 and KBG10

A cDNA library was established in λgt11 phage from mRNA of Kentucky bluegrass pollen(17-19). Three positive cDNA clones KBG60, KBG8.3 and KBG10, were selected with human sera from patients highly allergic to KBG pollen, and were isolated and purified as the recombinant phage DNA. It was found according to sequence analysis(19) that KBG60 is a full-length cDNA clone coding for an allergenic protein of 303 amino acid residues and KBG8.3 and KBG10 are C-terminal regions of KBG60. The recombinant phage DNAs containing these three cDNA molecules were isolated and used to transfect strain Y1090. According to the methods described by Maniatis et al(20), large amount of the cloned recombinant phage DNA was isolated and purified. The cDNA inserts of the clones in the recombinant phage DNA were obtained by digestion of the phage DNA with a restriction enzyme *EcoR I*. The cDNA inserts were separated from the phage DNA by gel electrophoresis in agarose gel containing ethidium bromide and purified by electro-elution of the insert DNA from the gel(20).

Synthesis of rKBG60, rKBG8.3 and rKBG10 in *E. coli*

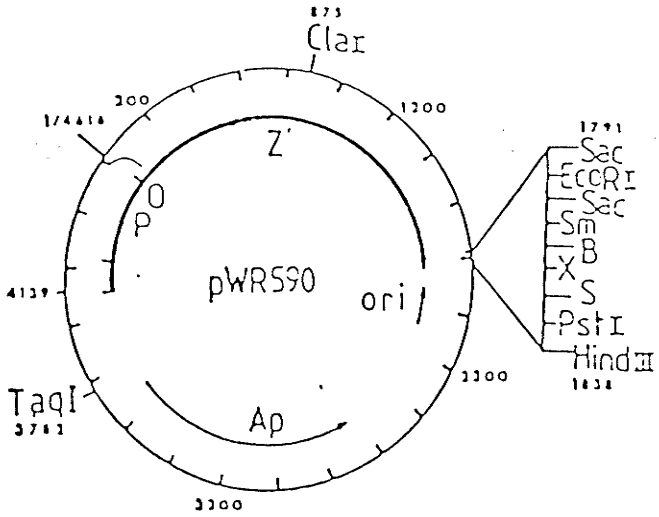
In order to obtain high-level expression of the recombinant allergens, the pWR590 series of expression plasmid vectors were used, which were kindly provided by Dr. D. Thomas(21). Based on the sequence data, pWR590.1 (Fig.1) was chosen for the synthesis of the recombinant allergens encoded by cDNA KBG60 and its fragments, KBG8.3 and KBG10. The

vector was digested with *EcoR I* and then its 5'-phosphate residues were removed with bovine pancreatic alkaline phosphatase(BRL, CA).

The cDNAs of KBG60, KBG8.3, and KBG10 with *EcoR I* ends were ligated into pWR590.1 with a cDNA insert to vector ratio of 4:1. T4 DNA ligase(BRL, CA) was used for the DNA ligation reaction. The recombinant plasmid vectors were transformed into and expressed in *E. coli* basically according to the method described before(22). Briefly, the recombinant DNAs were transformed into *E. coli* strain JM105 by using the standard $CaCl_2$ method(20). The transformed host cells were plated onto ampicillin-containing LB(Luria-Bertani: Bacto-tryptone 10g, Bacto-yeast extract 5g, NaCl 10g, per liter) agar plates. The colonies were first examined using 10% SDS-PAGE gel to look for the fusion proteins, the colonies found containing the fusion proteins were further examined with human IgE antibodies. The positives were then used to inoculate one liter of LB medium. The bacteria were grown overnight at 37^0C with consistent shaking.

Isolation and purification of the recombinant allergens

The recombinant KBG60 and its fragments synthesized in *E. coli* were isolated and purified with modified methods described before(22,23). Briefly, 1 liter of JM105 bacteria containing plasmid of the interest were harvested after overnight growth by centrifugation at 5,000rpm for 10 minutes. The pellets were washed with TE buffer(Tris 10mM, EDTA 1mM) pH7.4 twice, and then resuspended in 20ml of 50mM Tris buffer, pH9.5. These bacterial cells were subject to ultra-sonication, 15 seconds three times. The debris of the lysed cells were removed by centrifugation at



	582 583	
pWR590	Gly Asn	
	GCC AAC CGG GCG ACC TCG AAT TCG	
	582 583	
pWR590-1	Gly Asn	
	CGC AAC CGG GAG CTC G AA TTC	
	598 599	
pWR590-2	Asp Arg	
	GAT CGC GGG CCA GCT CG A ATT CGA	

Fig. 2. Physical map of pWR590 and the DNA sequences at the junction of the truncated Lac Z gene. The vector contains the lac promoter (P), operator (O) and the coding sequence for approximately 590 amino acids of Lac Z. The vector contains also an ampicillin-resistant gene (Ap) and a poly-linker. The junctional sequences of three reading frames of the vector family are shown below the vector map. The small arrow shows the location of the Eco RI site.

10,000rpm for 20 minutes. The supernatant was transferred into dialysis tubing with the molecular weight cut-off 12,000-14,000 daltons (Spectrum Medical Industries, Inc., CA). The supernatant was then dialysed against two liters of PBS buffer, pH7.0 overnight at 4^0C. The precipitate after dialysis was harvested by centrifugation at 10,000rpm for 10 minutes, and resuspended in 5ml SDS-PAGE loading buffer. The recombinant proteins were then subjected to SDS-PAGE gel and separated from the other co-precipitated proteins by electrophoresis. The region of the gel containing the recombinant protein was cut out after a small piece of the gel was stained with 1% Coomassie blue in 10% acetic acid and 20% methanol for visualization. The gel was smashed in a tube containing 10mM Tris.HCl buffer, pH9.5. The proteins were eluted from the gel by incubation at 37^0C overnight with vigorous shaking. The protein solution was dialysed twice against 10mM Tris buffer, pH9.5, and then lyophilized and stored at -20^0C.

Generation and synthesis of rKBG60.1 and rKBG60.2

For analysis of the N- and C-terminal regions of the rKBG60, a strategy of 3' region deletional mutation was used. The cDNA inserts, KBG60 and KBG8.3, were first ligated in pWR590.1 expression vector as described above. According to the restriction map of the inserts and the vector, the *Sal*I restriction enzyme was chosen to remove the 3' regions of cDNA60 and cDNA8.3 and the remaining segment was allowed to self-ligate to generate cDNA clones KBG60.1 and KBG60.2. The cDNA KBG60.1 codes for a polypeptide representing the N-terminal fragment of rKBG60, whereas KBG60.2 encodes a polypeptide representing the overlapping

region of rKBG60.1 and rKBG8.3. The expression plasmids containing KBG60.1 and KBG60.2 were transformed into JM105 for synthesis of the recombinant polypeptides, which were then isolated and purified using the procedures described above.

Analysis of the conditions for the level of expression of the recombinant allergens

The factors affecting the optimal expression of one recombinant allergenic polypeptide, rKBG8.3, were analyzed. First, the effect of incubation time on the expression of the recombinant polypeptides was examined. Twenty millilitre of LB medium, pH7.5, was inoculated with 10μl of the overnight hour culture and incubated at 37^{0}C for different periods of time, e.g., 3h, 6h, and overnight hour. The second factor investigated was pH effect. The bacteria were grown overnight in media with pH in the range of 5.5 to 8.5, with 0.5 interval. The third one was the effect of IPTG(iso-propyl-β-thiogalactoside, BRL, CA), with the concentration of IPTG ranging from 0.1 to 0.5mM, on the induction of the expression of the overnight culture. Finally, the combined effect of growth time and IPTG on the expression at pH7.5 was observed. The level of each expression of the recombinant allergenic polypeptide was determined by SDS-PAGE gel. The proportion of each expression of the recombinant allergen in the bacterial lysate was analyzed with Quick Scan R & D electrophoresis TLC densitometer (Helena Laboratories, Texas).

SDS-PAGE and Western blotting analysis

SDS-PAGE was performed according to Laemmli(24) to examine the expression of the recombinant proteins. The bacteria containing the recombinant proteins were lysed with SDS-PAGE loading buffer (Tris.HCl 0.0625M, pH6.8, Glycerol 10%, 2-ME 5%, SDS 2.5%) by boiling for 5 minutes. The proteins were then loaded onto 10% polyacrylamide gel in a slab-gel apparatus and separated by electrophoresis, followed by staining with Coomassie blue in 10% acetic acid and 20% methanol or electrotransferred onto nitrocellulose membranes for immunoassay. The membranes were first blocked with 1% gelatin in PBS buffer and then incubated with 1 in 10 diluted human sera. The bound IgE antibodies were detected by enzymatic color reaction with 1:2000 diluted goat anti-human IgE conjugated with alkaline phosphatase(TAGO, CA) and its substrate, *p*-nitro tetrazolium chloride (NTB) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP), in pH9.6 alkaline phosphatase (AP) buffer containing Tris 0.1M, NaCl 0.1M, $MgCl_2$ 5mM.

For determination of the KBG grass pollen proteins corresponding to the recombinant allergen, murine anti-rKBG8.3 sera were employed in Western blotting analysis. Ten microgram of KBG pollen extract prepared as previously described(25) was loaded on a 15% polyacrylamide gel and the proteins were separated by electrophoresis. The separated proteins were either stained with Coomassie blue or electroblotted onto a nitrocellulose membrane. The membrane was blocked with 1% gelatin in PBS buffer and probed with 1 in 2000 diluted anti-rKBG8.3 sera. The immunoreactive bands were then detected using an alkaline phosphatase conjugated goat anti-murine Ig(Zymed, CA) and substrate NTB and BCIP in AP buffer.

Human sera used for screening and immunoassays

Sera were collected from patients highly allergic to grass pollens as determined by skin-prick test. ELISA assay was employed to examine the KBG pollen-specific IgE titers of these patients' sera. Eleven individual sera with the highest titer of specific IgE to KBG pollen extract were pooled. Another serum pool from five individuals with undetectable IgE antibodies to KBG pollen was used as control. These serum pools were aliquotted and stored at -20^{0}C.

Immunization of mice

Female BDF1 mice(Jackson Laboratories, Bar Harbor, MI), 6-8 weeks old, were immunized subcutaneously in nuchal region with KBG pollen extract and rKBG8.3 polypeptide, respectively. The amount of the extract and the recombinant protein used for immunization was 20μg and 5μg per mouse. For the respectively primary immunization, 2.5 mg of dextran sulfate per mouse was used as an adjuvant. Twenty-three days later the same amount of proteins and 1mg dextran sulfate was used to boost the mice in the other side of the nuchal region. The mice were bled after 7 days of boosting and the sera were collected and stored at -20^{0}C.

Analysis of the antibody-binding activities of the recombinant KBG60 and its fragment

Allergenicity of the recombinant polypeptides was analyzed using an enzyme-linked immunoassay. For this assay, 0.1μg of the purified recombinant polypeptides or the truncated β-galactosidase, and 1μg of KBG pollen extract, determined by Bio-rad assay, in 100μl

carbonate/bicarbonate, pH9.6, buffer were used to coat each well of a microtiter plate (Nunc, Denmark) overnight, followed by blocking with 2% gelatin in PBS buffer for 2 hours at room temperature. Each of the two pools of human sera from allergic or non-allergic individuals at a 10-fold dilution in PBS dilution buffer (containing 0.5% BSA and 0.1% Tween 20) was then added to the wells and the plates were incubated at room temperature overnight. The bound IgE antibodies were detected by using 1:2000 diluted alkaline phosphatase-conjugated goat anti-human IgE antibodies(TAGO, CA) and the substrate *p*-nitrophenyl in AP buffer for enzymatic color reaction at 37^0C for 30 minutes.

The ability of two fragments, rKBG60.1 and rKBG8.3, which represent the N- and C-terminal regions of the full-length allergen rKBG60, to bind IgE antibodies was examined by employing the serially diluted serum pool of the allergic individuals. The relative proportions of the IgE antibodies directed to the recombinant polypeptides were estimated by using the absorption method. Briefly, 1mg of the insolubilized form of each of rKBG60, rKBG8.3 and rKBG60.1 was incubated with the human sera 1:40 diluted with the PBS dilution buffer for four hours at room temperature. The polypeptide-bound IgE antibodies were then removed by centrifugation of the complexes at 10,000 rpm for 10 minutes. These absorbed sera were then examined for residual IgE antibodies by ELISA using wells coated with KBG pollen protein extract, 1μg per well, or the recombinant polypeptides, 0.1μg per well. Murine anti-KBG antisera (1:2000) were also employed to determine the antigenicity of the fragments with the above procedure, except the second antibody used was alkaline phosphatase-conjugated goat anti-mouse IgG(Zymed, CA).

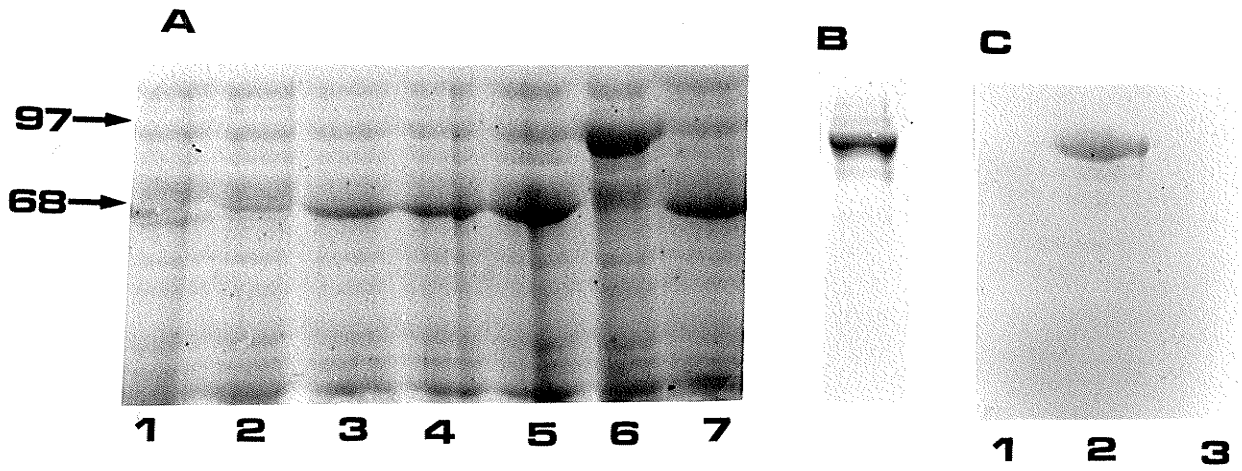


Fig. 3. Identification of transformants containing recombinant allergenic polypeptide rKBG8.3. The bacterial lysates of ampicillin-resistant colonies were first examined using SDS-PAGE (3A). The purified rKBG8.3 was shown in 3B. The proteins presented in lane 6 and its two adjacent lanes in 3A were transfered onto nitrocellulose membrane and probed with human IgE antibodies in 3C.

RESULTS

Synthesis of the recombinant allergens in *E. coli*

The expression, purification, and IgE-binding analysis of a recombinant allergenic polypeptide, rKBG8.3, is presented in Fig.3. Among seven ampicillin-resistant colonies (Fig.3A), the bacterial lysate from one of them was found to contain the fusion protein (lane 6), whereas the lanes 2-5 and 7 possessed only the truncated β-galactosidase. Analysis of the plasmid DNAs from these clones led to the finding that the plasmid DNAs from two colonies represented in lane 1 and lane 6 possessed cDNA insert of KBG8.3 although only one of them expressed the fusion protein. The recombinant polypeptide was purified and analyzed for its ability to react with human IgE antibodies. Fig.3B shows the purified rKBG8.3 from the bacterial lysate. It was obvious that with the above purification procedure most of the bacterial proteins could be removed. The recombinant protein was recognized by human IgE antibodies whereas the adjacent lysate containing only the truncated β-galactosidase and other bacterial proteins showed no reactivity with human IgE antibodies (Fig.3C).

With a similar procedure, a total of five overlapping recombinant allergenic polypeptides were synthesized in *E. coli* (Fig.4). These recombinant allergenic polypeptides included a full length protein rKBG60, its one N-terminal fragment rKBG60.1, two C-terminal fragments rKBG8.3 and rKBG10.0, and a small fragment rKBG60.2 representing the overlapping region of the rKBG8.3 and the rKBG60.1. The lengths and

relative positions of these polypeptides in rKBG60 are schematically shown in Fig.5. The molecular weights of the recombinant polypeptides were consistent with the lengths of the polypeptides deduced from the sequence of the cDNA inserts with the exception of the polypeptide rKBG60.1, which seemed to be of higher molecular mass than its deduced size.

It was observed that the expression levels of the recombinant polypeptides were different, ranging approximately from 15% to 40% of total cell proteins as determined by using densitometry (Table 1).

Table 1. Expression levels of the recombinant polypeptides

recombinant polypeptides	percentage of the total bacterial proteins
rKBG60	18.9
rKBG60.1	16.3
rKBG8.3	39.5
rKBG10	38.2
rKBG60.2	40.7

The clones of rKBG8.3, rKBG10.0 and rKBG60.2 had relatively higher expression levels compared to those of the rKBG60 and the rKBG60.1 (Fig.4). The factors possibly affecting the expression levels of the rKBG8.3 included the incubation time, pH value of the medium and IPTG

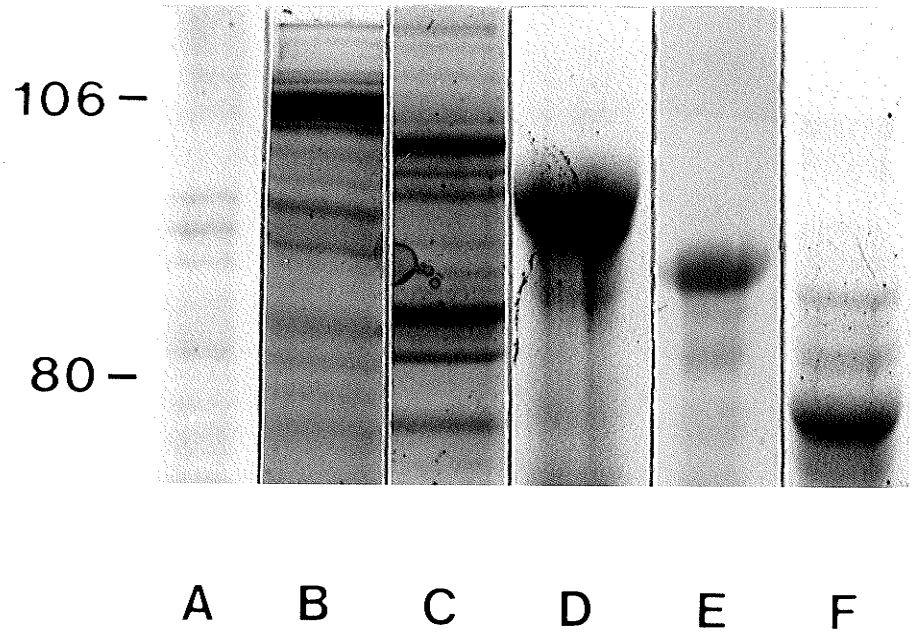


Fig. 4. Synthesis of rKBG60 and its four overlapping fragments. The fusion proteins were electrophoresed on SDS-PAGE gel. The lanes containing the different proteins from the bacteria are marked with letters as: non-transformed JM105 cell lysate (A); rKBG60 (B); rKBG60.1 (C); rKBG8.3 (D); rKBG10 (E); rKBG60.2 (F).

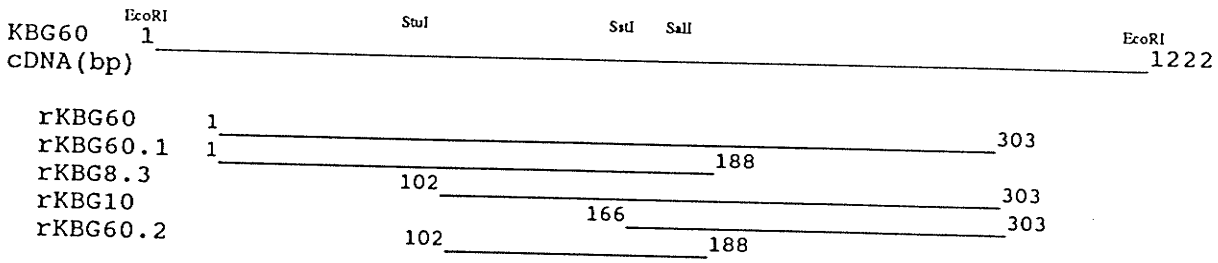


Fig. 5. Map of a full-length of allergen, rKBG60, and its four overlapping fragments. The cDNA clones KBG60, KBG8.3 and KBG10 were found from λgt11 library using human IgE antibody as probe whereas KBG60.1 and KBG60.2 were generated from KBG60 and KBG8.3, respectively.

induction. Although the expression of the recombinant protein with the plasmid vector pWR590.1 is associated with *Lac Z* gene, IPTG in our system seemed to have no effect on the induction of the recombinant polypeptide regardless of the time of its addition, i.e., at the start of the culture, the log phase, or the stationary phase. The pH value of the medium ranged from 5.5 to 8.5 and was found to have no effect on the level of expression either. The only factor apparently affecting the expression was the time of the incubation. Our results indicated that the expression of the recombinant polypeptide could be achieved at the stationary phase of the culture. Therefore, all of the recombinant polypeptides were obtained in this system from the overnight culture.

Binding of IgE antibodies by recombinant fragments of rKBG60

The full length allergenic protein, rKBG60, and its four overlapping fragments in association with the truncated β-galatosidase purified from the bacterial lysate were examined for their abilities to bind human IgE antibodies by using a pool of 11 sera of individuals allergic to KBG pollen, as well as another serum pool of 5 individuals not allergic to the pollen as a negative control. It was found that all the recombinant polypeptides were able to bind human IgE antibodies, but to different degrees (Fig.6). KBG pollen extract and the truncated β-galactosidase isolated from the self-ligated transformants were employed as positive and negative controls, respectively. These results were confirmed by radioimmunoassay (data not shown) and suggested that human IgE antibody binding epitopes were localized on all four polypeptides of the rKBG60 allergenic molecule.

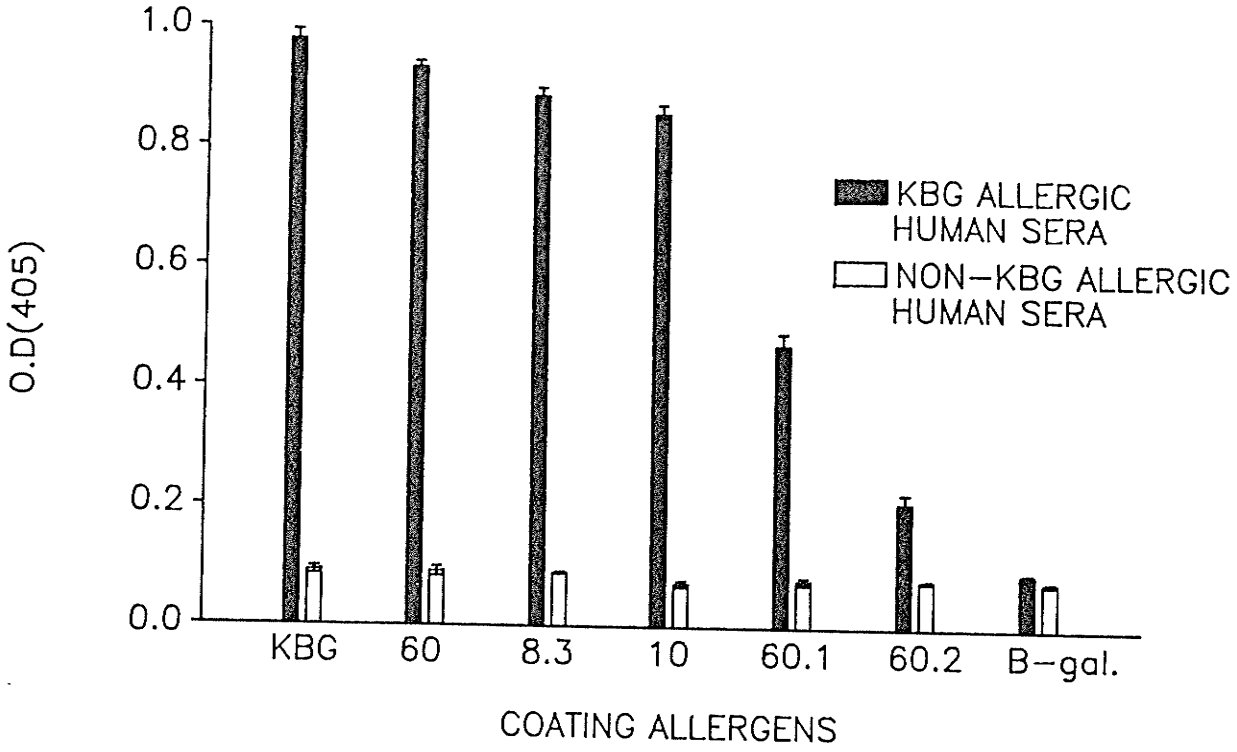


Fig. 6. Human IgE bindings of rKBG60 allergen and its fragments. Human IgE binding assay was performed by ELISA. Non-KBG allergenic human sera were used as control, moreover KBG pollen extract and partial β-galactosidase isolated from the pWR590.1 transformed bacteria in the same way as the recombinant proteins were used as positive and negative control, respectively. All fragments show more or less IgE bindings.

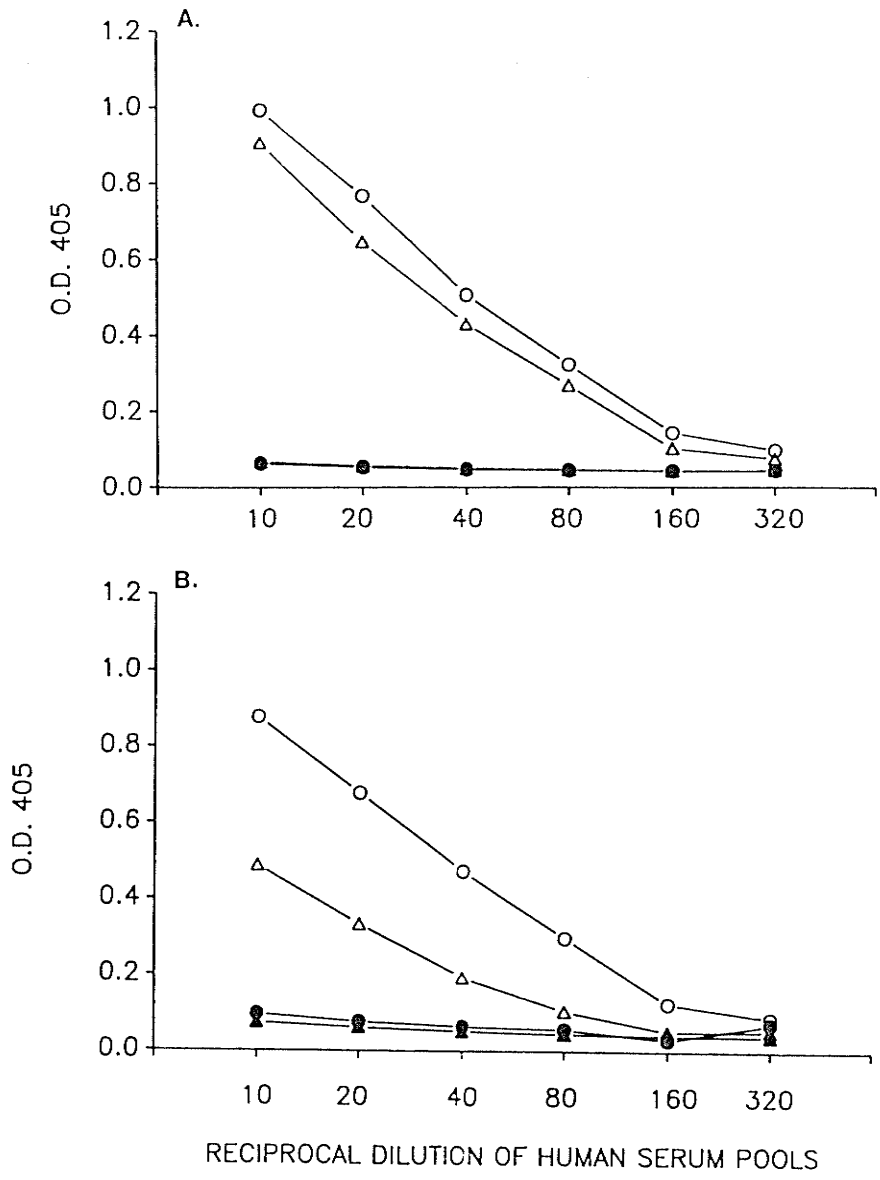


Fig. 7. Serially diluted human serum pools from KBG-allergic patients and KBG non-allergic individuals are examined for their specific IgE titers against the recombinant allergens by ELISA. The reactivities of rKBG60(○-○) and KBG extract(△-△) to the atopic serum pool and rKBG60(●-●) and KBG extract(▲-▲) to the non-atopic serum pool are shown in 7A. The reactivities of the fragments of rKBG8.3(○-○) and rKBG60.1(△-△) to the atopic serum pool and rKBG8.3(●-●) and rKBG60.1(▲-▲) to the non-atopic serum pool are shown in 7B.

Distribution of human IgE binding epitopes of rKBG60

The distribution of IgE-binding epitopes of the rKBG60 was analyzed by using the two recombinant fragments, rKBG60.1 and rKBG8.3 representing respectively N-terminal and C-terminal regions. The reactivities of the rKBG60, rKBG60.1 and rKBG8.3 to IgE antibodies were further assayed by ELISA using the serum pools. The binding activities of the sera to the allergens showed typical dilution-dependent curves (Fig.7A,B). The results indicated that the sera contained high levels of specific IgE antibodies not only to KBG pollen extract but also to the rKBG60 allergen. The IgE binding of the rKBG8.3 was similar to that of the rKBG60 in terms of its O.D readings. However, the rKBG60.1 was much lower in the binding than both rKBG60 and rKBG8.3, although its peptide-chain length is similar to that of the rKBG8.3.

To confirm the distribution of the IgE binding epitopes of the rKBG60 allergen, an absorption assay was employed (Fig.8). It was found that the rKBG60 allergen could remove about 20% of IgE antibodies specific to the whole extract of KBG pollen. This absorbed sera when employed for western blotting showed no detectable IgE binding to *Poa p* IX allergens whereas the IgE antibodies specific for allergens belonging to other groups remained unaffected (not shown), indicating that the recombinant allergen retained its IgE antibody binding activity comparable to the corresponding native allergen in the grass pollen. In a similar manner, it was demonstrated that the C-terminal part of the molecule, the rKBG8.3, could remove about 83% of total IgE binding activity directed to the full-length rKBG60 allergen. By contrast, the N-terminal fragment, rKBG60.1, removed only 32% of the IgE binding

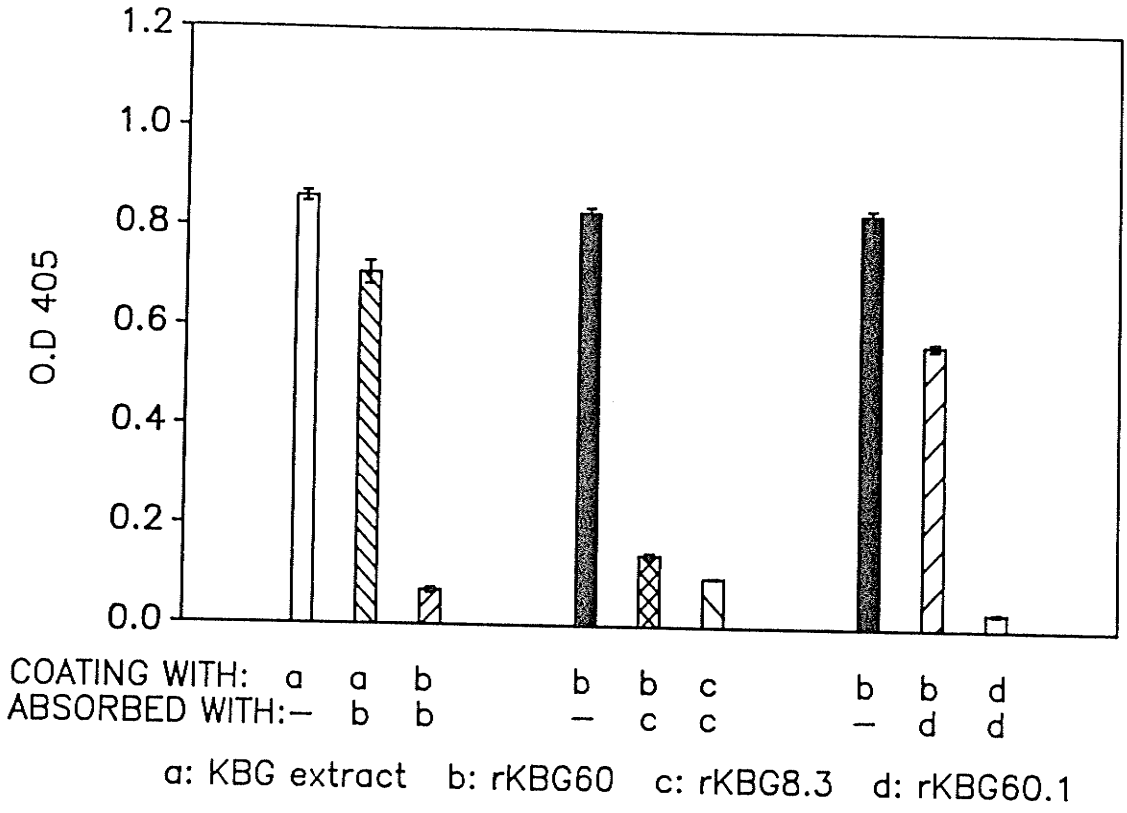


Fig. 8. Distribution of IgE-binding epitopes in the rKBG60 allergen. The serum pool from individuals allergic to KBG pollen was absorbed with rKBG60, or its fragments rKBG8.3 and rKBG60.1. The absorbed sera were then applied to the wells coated with appropriate antigens as described and analyzed for remaining IgE antibody levels corresponding to the recombinant allergens.

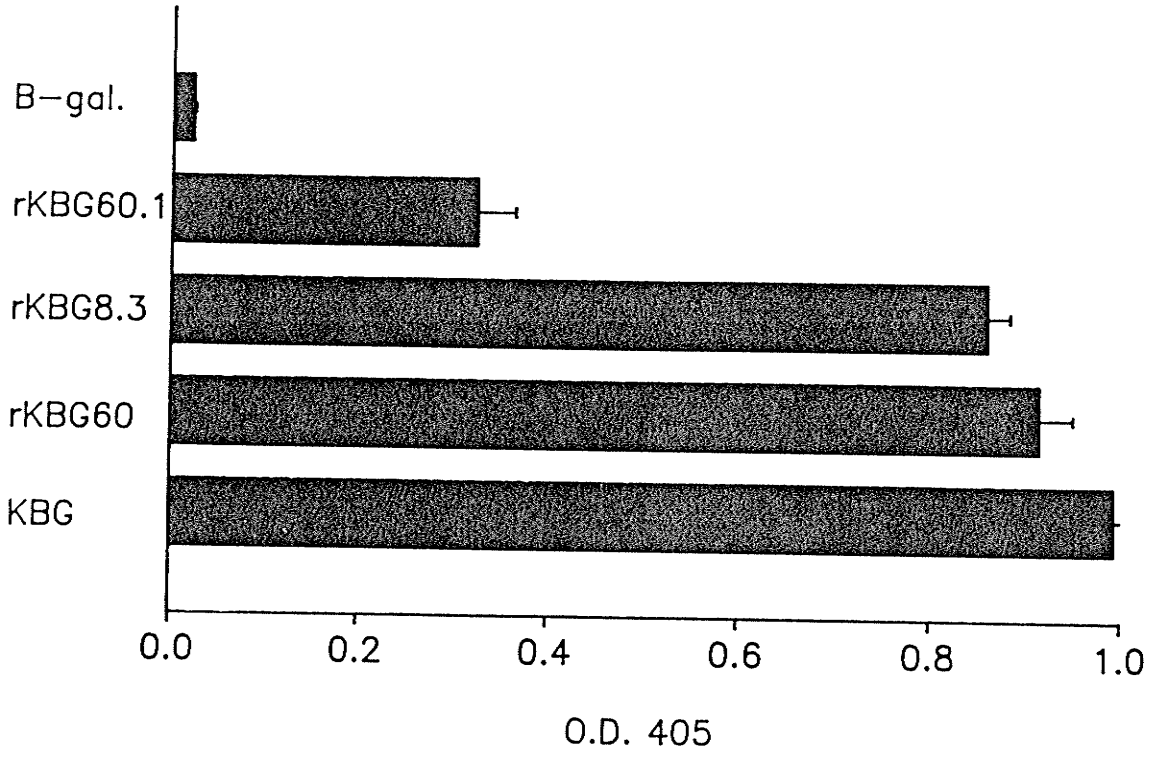


Fig. 9. Murine anti-KBG antibody binding to rKBG60 and its two fragments. ELISA was performed to detect the antibody specific to the recombinant KBG60 and the fragments in the antisera raised against extract of KBG pollen.

activity. When both rKBG8.3 and rKBG60.1 were used at the same time to absorb the sera, the IgE antibody reactivity to the rKBG60 allergen was removed completely. These results suggested that the major IgE binding epitopes locate on the C-terminal region of the recombinant allergenic protein rKBG60.

Reactivity of the rKBG60 allergen to murine anti-KBG antibodies

The rKBG60 allergen and its two terminal fragments were analyzed by ELISA for their ability to bind murine anti-KBG antibodies. As is obvious from the results illustrated in Fig.9, the three recombinant products, rKBG60, rKBG60.1 and rKBG8.3 were recognized by anti-KBG antibodies. Thus, it provided the evidence that KBG pollen possessed allergenic proteins equivalent of the recombinant allergen. Whereas the fragment rKBG8.3 approached the reactivity of the full-length molecule, i.e., the rKBG60, the reactivity of the rKBG60.1 did not exceed one third of that of the rKBG60. Hence, these results support the view that most of the antibody binding sites were located in the C-terminal region of the rKBG60.

Identification of three major isoallergens in KBG pollen corresponding to the recombinant allergens

Western blotting was employed to determine the proteins in KBG pollen which correspond to the recombinant allergen. The results presented in Fig.10 demonstrated that the murine antibodies raised against the conserved region of our cloned isoallergens, rKBG8.3, recognized three bands of KBG pollen proteins, with molecular weight of 28KDa, 32KDa and 34KDa, respectively. Thus, the originality of the

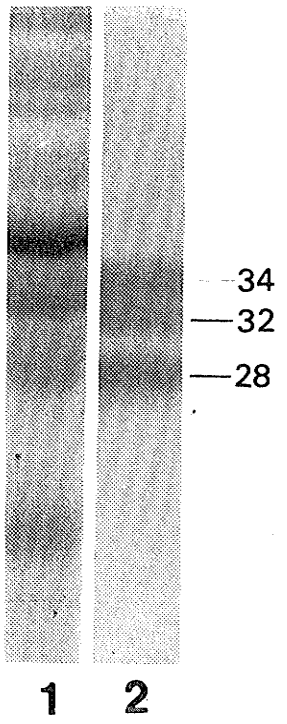


Fig. 10. Western blotting analysis of the recombinant allergen-natural proteins. The KBG pollen proteins were electrophoresed on SDS-PAGE and stained with Coomassia blue (lane 1) or electroblotted onto nitrocellulose membrane. The membrane was probed with mouse anti-rKBG8.3 (lane 2). The numbers on the right indicate the molecular mass estimated in KDa.

recombinant allergens from KBG pollen was confirmed and the corresponding native allergens in the grass pollen were defined in terms of their molecular sizes.

DISCUSSION

Gene cloning and expression techniques permit the production of pure proteins in unlimited amounts from cloned cDNA molecules. Recent application of these techniques in allergen studies have led to the synthesis of several common allergens(14-16). In the present study, a full-length recombinant allergen, rKBG60 and its four overlapping fragments were synthesized by employing a plasmid vector, pWR590.1, in *E. coli*. The expression system made it possible to obtain large amounts of purified recombinant allergenic polypeptides. Although all the recombinant polypeptides were expressed in the form of fusion proteins, i.e., in association with a truncated β-galactosidase, their immunological reactivities in terms of recognition by human IgE and murine IgG antibodies and induction of KBG-specific antibodies *in vivo* appear not to be dramatically affected. Thus, synthesis of immunologically active allergens of grass pollens in large amounts was achieved in this study by using the recombinant DNA procedures.

The advantage of using the pWR590.1 vector to obtain high level expression of stable fusion proteins in *E. coli*(21), in particular for the low molecular weight polypeptide, was also observed in our system. The smallest KBG-origin fragment we synthesized was the 66 amino acid peptide rKBG60.2, where the amount of the fusion protein was up to 40%

of the total cell proteins. Under the identical conditions, the similar level of expression of the rKBG8.3 and the rKBG10 had been achieved, whereas the expression levels of the rKBG60 and the rKBG60.1 were relatively low. Similar results have been obtained in another expression system, i.e., pGEX system (Olsen & Mohapatra, 1992); however, the reasons for such differential expression are not known at present.

Although the pWR590 vector has the regulatory sequences of *Lac Z* gene, IPTG appeared not to be effective on release of the *Ic* repressor and induction of the expression of the fusion protein. Instead, the expression of the fusion protein was closely related to the incubation time or the growth stage of the bacteria. The results from this study are consistent with the initial report from Guo et al (21) that the fusion protein expressed highly when the bacteria reached the stationary phase. This is probably due to the slow accumulation of the fusion protein in the cells. Moreover, other regulatory elements, such as the lack of appropriate regulation of the *Ic* gene of the *lac* operon, which may contribute for the low-level expression of the fusion protein when the bacteria are in the logarithmic phase of growth. Nevertheless, the system used in this study for generation of the allergenic fragments is relatively simple, less expensive, and easily manipulated.

A major finding of this study was that the C-terminal region is the most allergenic/antigenic portion of the rKBG60 allergen. The evidence presented has clearly shown that this region of the molecule binds more than 80% of the antibodies specific for the entire molecule of rKBG60 allergen. These observations are consistent with our previous predictions from analysis of the primary structure of the rKBG60 that

the most conserved C-terminal region possessed the greatest potential for antigenicity (19,22). Furthermore, these results are similar to the observations of another cloned grass pollen allergen, referred to as *Lol p* Ib (26), of which only the C-terminal fragment bound IgE antibodies. Indeed, a comparison of the deduced sequence of this *Lol p* Ib allergen with that of the rKBG60 indicated about 56% homology between the conserved C-terminal regions of these allergens.

It is to be noted, however, that in our study the N-terminal region of the rKBG60 was not completely devoid of allergenicity. This region appeared to be less allergenic and antigenic as revealed by binding patterns with the human and murine antibodies, respectively. Similarly, the overlapping part of the N- and C-terminal fragments, represented by the rKBG60.2, was also shown to bind human IgE antibody albeit the binding was relatively weak. Interestingly, there was also very weak murine antibody binding activity of this fragment when the anti-KBG pollen extract was employed (data not shown). It is difficult, however, based on these data to estimate its actual antibody binding ability because of the possibility that this smaller fragment may not be exposed fully on the surface of the fusion protein. Furthermore, since the rKBG8.3 could not remove all the IgE-binding epitopes from the rKBG60 and the rKBG60.2 showed much weaker IgE binding than the rKBG60.1, it can be concluded that there are some other IgE-binding epitopes in the N-terminal region upstream of the rKBG8.3 or the rKBG60.2.

In addition, it should be pointed out that in this context of the ELISA the O.D. values for the KBG extract were not directly comparable to those for the recombinant polypeptides since the extract comprised

numerous components. The majority of these components are not allergenic proteins, nevertheless, they compete with the allergens with respect to binding sites on the ELISA plates. Hence, although the KBG extract may be viewed as a positive control, a direct comparison between the extract and the recombinant polypeptides in terms of their ability to bind IgE antibodies is not possible.

Moreover, the results presented in this study demonstrated only the distribution of antibody binding epitopes but not the detailed locations and the numbers of the epitopes on the rKBG60. Further analysis of the epitopes have to employ the smaller recombinant or synthetic peptides. From these data it can be concluded that the recombinant fragments of the allergen still retained their antibody binding capacities. These data in conjunction with the evidence that there is no cysteine in this full-length allergenic molecule, and therefore no major intrachain S-S bond formation, suggest that some, if not all, antibody binding epitopes be mainly sequential in nature. Thus, the synthetic peptides will be suitable for the epitope mapping of this allergen.

For identification of the native protein corresponding to the polypeptides encoded by the cDNA clones, techniques such as hybrid-select translation followed by immunoprecipitation is usually employed. Because of the low concentration of IgE antibodies in the patients' sera and also of the corresponding mRNA and native proteins in the grass pollen, it is difficult to identify the corresponding native proteins by using the above procedure. Availability of the recombinant allergenic polypeptides enabled us to generate the specific antibodies, with which three protein bands were precisely identified.

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CHAPTER III

ANALYSIS OF ANTIBODY-BINDING AND -INDUCING EPITOPES OF THE rKBG60 ALLERGEN

ABSTRACT

For delineation of the antibody-binding epitopes of the recombinant KBG60 allergen, 56 decapeptides with 5 amino acid overlaps were synthesized on polypropylene pins. To confirm the results of the epitope mapping obtained with the peptides on the pins and to analyze further human IgE epitopes, another set of 23 peptides with 20 residues in length and 10 amino acid overlapping were also synthesized. By probing the synthetic peptides with the murine antisera raised against KBG pollen extract, the rKBG60 and the rKBG8.3, at least 13 murine antibody-binding epitopes were defined. One of these epitopes spanned by the decapeptides #46-#53 or the 20 residue peptides #26-#28 was found only when the 20 residue peptides were used, indicating the lengths of the peptides influenced the detection of this epitope. Furthermore, employment of human serum pools to examine the free 20 residue synthetic peptides led to demarkation of at least 6 IgG binding epitopes and 4 IgE binding epitopes of the rKBG60 allergen. The majority of the human IgE binding peptides were also recognized by human IgG and murine antibodies, although some differences were observed. Moreover, the possibility that both B- and T-cell epitopes coincide on the same short peptide was examined by induction of antibody production *in vivo* with the synthetic peptides. The results demonstrated that the majority of the tested antibody-binding peptides also contained T cell epitopes.

INTRODUCTION

The development of therapeutic strategies for allergic diseases that would directly and specifically regulate IgE antibody production to defined allergens requires detailed knowledge of the structure of individual allergens, in particular, of their epitopes, which are recognized by antibodies and T cells. Similar to other protein antigens, the epitopes of allergens may be also sequential (or linear) and conformational(1). Although recent studies with X-ray crystallography demonstrated that all antibody binding epitopes are conformational in nature(2), various stabilities of epitopes of different allergens when treated with denaturing agents indicate that some IgE binding epitopes are more sequential while the others are more conformational(3-6). Conformational epitopes are usually more easily destroyed by denaturing agents and require the intact allergens or large allergenic fragments to be analyzed, whereas sequential epitopes are stable regardless of usage of denaturing agents and can be demarcated by employing synthetic peptides.

The methods utilized for identification of linear epitopes consisted of probing recombinant polypeptides or synthetic peptides of the antigen with specific antibodies. Several investigators have employed recombinant DNA procedures which involve expression of a series of overlapping cDNA clones followed by antibody screening for the delineation of linear epitopes(7-10). Atassi and his associates(11), on the other hand, employed a comprehensive

overlapping peptide synthesis strategy to delineate the epitopes of a ragweed pollen allergen, *Amb a* III, which bound to IgE and IgG antibodies. Recently, chemically synthesized peptides were also used for the identification of B cell epitopes of the major mite allergens(12), codfish allergen *Gad c* I(13), the chironomid hemoglobins(14), and wheat amylase inhibitor allergen(15). However, the structure of B cell epitopes of the grass pollen allergens, which constitute a major group of aero-allergens worldwide remain to be elucidated. This is primarily due to the lack of information on the structures of these allergens until recently.

The complete amino acid sequences of the two allergens, *Lol p* II(16) and *Lol p* III(17) were determined by sequencing of the purified proteins. Moreover, the primary structure of two *Lop p* I isoallergens were determined by the application of cDNA cloning and sequencing procedures(18,19). The *Poa p* IX allergens studied in our laboratory (20-23) have been primarily analyzed for their antibody-binding epitopes with the overlapping fragments (detailed in chapter II). Although it was demonstrated that the major antibody binding epitopes are on the C-terminal region of the protein, the location and the numbers of the epitopes of the rKBG60 have not been defined. In this study, we analyzed the epitopes of the rKBG60 with non-cleavable peptides on polypropylene pins and peptides synthesized with conventional procedures. Binding studies of these peptides with different antisera revealed that there are at least 13 murine antibody-binding epitopes on the rKBG60 allergen. Some of these epitope-containing peptides were also recognized by human IgG

and IgE antibodies, and were coincident with T-cell epitopes.

MATERIALS AND METHODS

Peptide synthesis and epitope mapping on the polypropylene pins

An epitope mapping kit(CRB, Cambridge, England) was used to synthesize overlapping peptides of the rKBG60 allergen according to the manufacturer's instructions. Since the amino acid residues 1-22 of the rKBG60 allergen were regarded as the leader sequence according to the analysis of the primary structures of the isoallergens(20), decapeptides with 5 amino acid overlap were designed starting with residue 23; e.g., the first decapeptide was made up of residues 23-32, the second was of residues 28-37, and so on to the end of the molecule. Altogether 56 peptides which spanned the remaining rKBG60 molecule and two other well-established peptides representing positive and negative controls were synthesized simultaneously. These peptides were synthesized by incorporation of the Fmoc amino acid activated esters onto polypropylene pins supplied with the kit, basically according to the method described by Geysen et al(24).

Murine anti-KBG extract, anti-rKBG60, and anti-rKBG8.3 antibodies were used to detect the antibody binding of the peptides. The antibody-peptide binding assay on the pins were performed in microtiter wells of ELISA plates. The pins were first blocked with 2% gelatin in PBS buffer in a microtiter plate at 37^{0}C

for 1 hour, and then incubated overnight with either normal murine, anti-KBG extract, anti-rKBG60, anti-rKBG8.3, or anti-β-galactosidase sera, in 1:100 dilution in PBS buffer containing 5% Tween-20 at 4^0C overnight. The bound murine IgG antibodies were detected by using HRP-conjugated sheep anti-mouse IgG (Biorad, ON) by incubation of the pins with the conjugate at 37^0C for one hour, followed by insertion of the pins into the wells containing substrate azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)(Sigma, MO). After a 30-minute incubation at room temperature to allow color development, the results of the reactions were read with ELISA reader(Dynatech Institute, Torrance, CA) and expressed as O.D. values.

Analysis of epitopes with the 20 residue synthetic peptides

For confirmation of the results of antibody-binding epitopes defined by using the synthetic peptides on the polypropylene pins and analysis of human IgE and IgG antibody-binding epitopes, 23 free 20 residue peptides with 10 amino acid overlaps were designed covering most regions of rKBG60 allergen. The peptides were synthesized by Dr. P. Chong of the Department of Protein Engineering, Connaught Laboratories Limited, Toronto, using a peptide synthesizer essentially based on the methods described by Merrifield(25). After cleavage, the peptides were purified by reverse-phase HPLC.

The binding of these peptides to murine anti-rKBG60, human IgE antibodies and human IgG antibodies, and anti-peptide sera was

examined by ELISA. Briefly, each well of Nunc Maxisorb (BRL, CA) microplate was coated with 10μg peptide in 100μl of 0.05M carbonate/bicarbonate buffer, pH9.6 overnight at room temperature. The plates were then washed with PBS-Tween buffer three times, and the free sites of the wells were saturated with 2% BSA in PBS buffer by incubation at room temperature for 2 hours, followed by addition of 100μl of either the murine antisera raised against rKBG60 at 1:100 dilution, or raised against the peptides at 1:20 dilution. For detection of human IgG- and IgE-binding peptides, 1:50 and 1:2 diluted human sera were used, respectively. The dilution buffer was PBS buffer containing 0.5% BSA and 0.1% Tween-20. The plates were incubated with the antisera at room temperature overnight, followed by three washes with PBS-Tween buffer. The bound antibodies were detected with alkaline phosphatase conjugated goat anti-mouse Ig (Zymed, CA), or goat anti-human IgG (Bio-rad, ON), or goat anti-human IgE (TAGO, CA). All the second antibodies were used at 1:2000 dilution. The colour development of the substrate was carried out at 37^{0}C for 60 minutes and the O.D. values were read with an ELISA reader.

Human sera:

The same serum pools used for the peptide binding studies were from 11 patients and 5 non-KBG atopic individuals as described in Chapter II.

Preparation of murine antibodies

The murine antibodies against KBG pollen and the recombinant allergens were produced as described in Chapter II. The antibody titer of anti-rKBG60 was up to 1:128,000, similar to that of anti-rKBG8.3, which will be presented in detail in the following Chapter.

The capacities of the antibody binding peptides to induce antibodies *in vivo* was examined by immunization of mice with the corresponding peptides. For this purpose, female BDF1 mice, 6-8 weeks old, were immunized with the synthetic peptides, 30μg per mouse in 2.5mg dextran sulfate. After a three-week interval, the mice were boosted three times with the same amount of peptides in 1mg dextran sulfate. The blood was collected after seven days of second, third and fourth immunization. The sera were isolated and stored at -20^{0}C to be assayed at the same time.

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

The specific antibodies induced by the peptides in mice were examined with ELISA. Microplates were coated with either 0.1μg of the rKBG8.3 protein or 10μg of the synthetic peptides per well in 100μl carbonate/bicarbonate buffer, pH9.6, overnight at room temperature. The plates were then washed three times and the empty sites were saturated with PBS buffer containing 2% BSA. The 1:20 diluted murine sera were added into the wells and incubated at room temperature overnight. The bound antibodies were detected with goat anti-murine Ig conjugated with alkaline phosphatase(Zymed, CA) and

the substrate of the enzyme was assayed by color reaction as described above.

RESULTS

Scanning of antibody-binding peptides synthesized on the polypropylene pins

In order to have a quick scan of the epitopes of rKBG60, 56 overlapping decapenta-peptides, covering the entire molecule, were synthesized. The peptides were examined with different antisera to define the antibody-binding epitopes.

Anti-KBG pollen extract: Scanning of the peptides for their ability to bind antibodies employing murine antibodies against native antigens of KBG pollen revealed several reactive peptides, #2, #4, #21-22, #31, #38, #41, and #44 (Fig.1A). The wells corresponding to these peptides exhibited O.D. readings above 0.3 which was more than the two-fold of the general background and negative control. The overlapping sequences of the adjacent positive peptides were considered as reactive regions. It is to be noted that for some peptides, for instance peptides #8-10, #12-15, #34, etc., it was difficult to determine their capacity to bind antibodies. The sera from the same mice before immunization showed

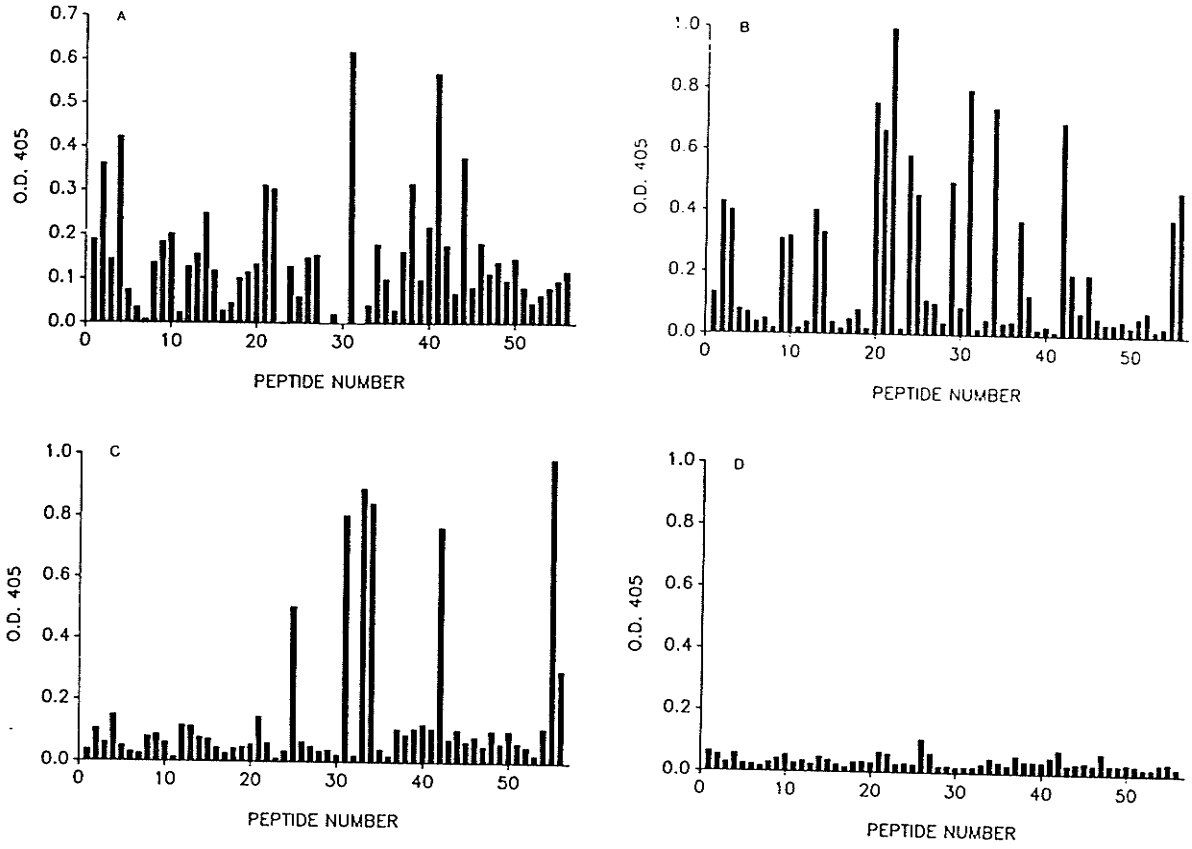


Fig.1 Murine antibody binding peptides defined with the 56 overlapping peptides on the polypropylene pins. The antibody-binding peptides were determined with ELISA. The sera used to recognized the epitopes were indicated as: a, anti-KBG; b, anti-rKBG60; c, anti-rKBG8.3; d, anti-β-gal.

no binding whatsoever to these peptides.

Anti-rKBG60: Antibodies induced with the full-length recombinant allergen, rKBG60, was also used to scan this series of peptides. Relatively lower background was observed compared to the scanning with anti-KBG pollen extract, therefore, resulting in better resolution. The reactive peptides revealed by this antisera were: #2-3, #9-10, #13-14, #20-22, #24-25, #29, #31, #34, #37, #42, #55-56 (Fig.1B). These 11 peptides, although showing different binding capacities as determined by O.D. values, were clearly above at least two-fold of the general background. The sera from the same mice before immunization reacted with none of these peptides.

Anti-rKBG8.3: Murine specific antibodies to the rKBG8.3 were also used to map the antibody-binding peptides. Five epitopes represented by peptides #25, #31, #33-34, #42, and #55 were demonstrated (Fig.1C). These peptides are all in the rKBG8.3-covered region of the rKBG60. The sera from the same mice before immunization showed no binding to the peptides.

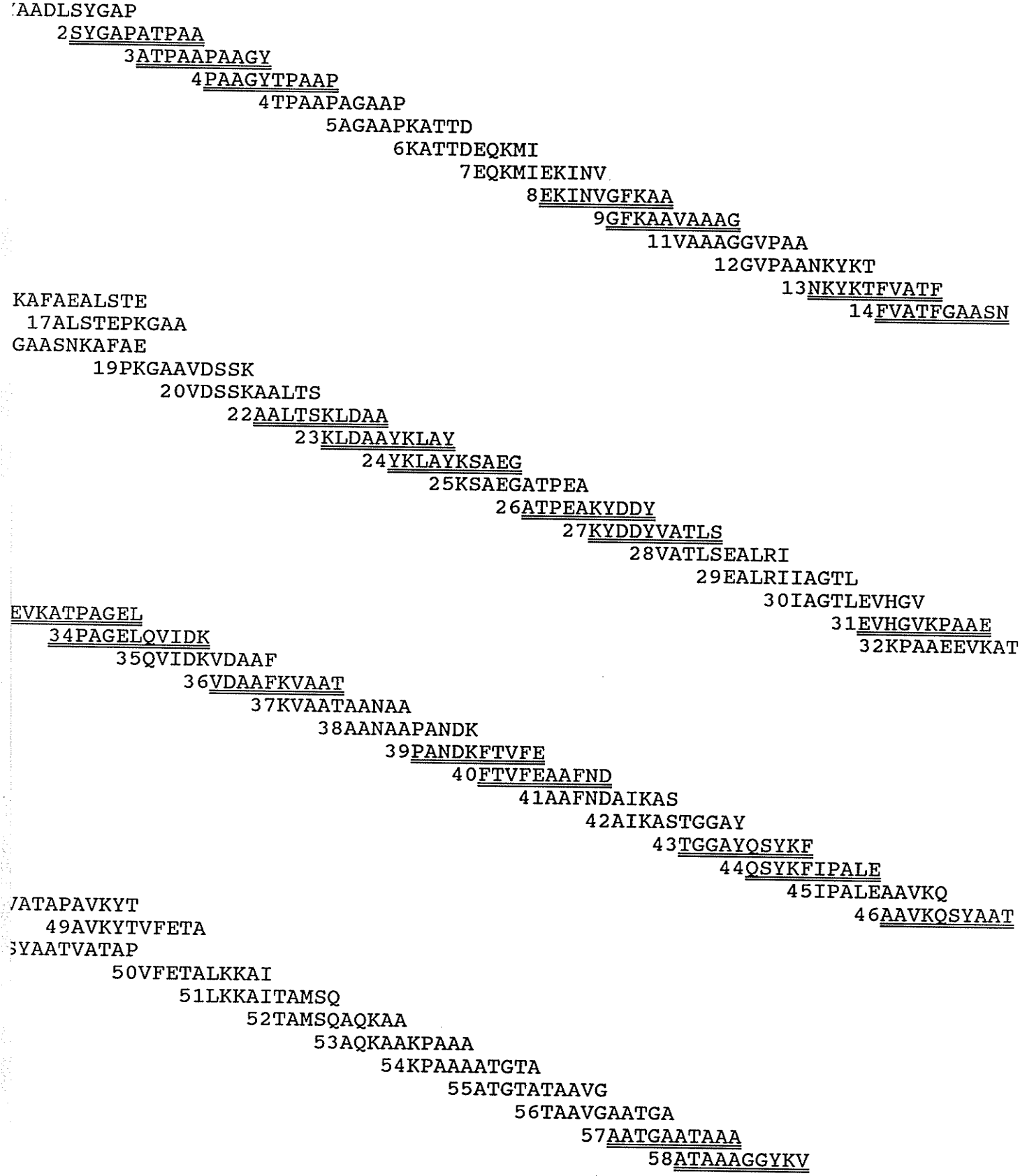
Anti-β-gal.: Since both of anti-rKBG60 and anti-rKBG8.3 sera contained antibodies to β-galactosidase, murine anti-β-galactosidase sera were used as control. It was clearly shown no binding of the anti-β-gal. to the peptides, therefore concluded that the antibodies bound to the peptides had no relevance to β-gal. (Fig.1D). All the results from the sera before immunization were similar to that of the anti-β-gal.

Table 1. The sequences of the peptides recognized by different antisera

Peptide number	Antisera			Amino acid sequences
	KBG	rKBG60	rKBG8.3	
2	+	+	*	SYGAPATPAA
3	-	+	*	ATPAAPAAGY
4	+	-	*	PAAGYTPAAP
9	±[a]	+	*	EKINVGFKAA
10	±	+	*	GFKAAVAAAG
13	±	+	*	NKYKTFVATF
14	±	+	*	FVATFGAASN
20	-	+	*	AALTSKLDAA
21	+	+	*	KLDAAYKLAY
22	+	+	*	YKLAYKSAEG
24	-	+	-	ATPEAKYDDY
25	-	+	+	KYDDYVATLS
29	-	+	-	EVHGVKPAAE
31	+	+	+	EVKATPAGEL
33	-	-	+	QVIDKVDAAF
34	±	+	+	VDAAFKVAAT
37	±	+	-	PANDKFTVFE
38	+	-	-	FTVFEAAFND
41	+	-	-	TGGAYQSYKF
42	-	+	+	QSYKFIPALE
44	+	-	-	AAVKQSYAAT
55	-	+	+	AATGAATAAA
56	-	+	±	ATAAAGGYKV

*: The N-terminal peptides not encompassed by the rKBG8.3.
[a]: The peptides difficult to evaluate the Ab-binding capacity.

)-BINDING PEPTIDES DEFINED BY MURINE ANTISERA



).2 Amino acid sequences and locations of the peptides recognized by the 'ine antibodies. The peptides recognized by antibodies are underlined.

With these three different sera, at least 12 epitopes were defined. Although some diversity was observed among the epitope maps obtained by these three sera, 8/12 for anti-rKBG60 versus anti-rKBG8.3 matches were observed(Table 1). Except peptides #24-25, #29, #44, and #55-56, the others mapped by anti-KBG pollen extract and anti-rKBG60 were well matched. Within the rKBG8.3 region 5 out of 6 epitopes, #24-25, #31, #33-34, #42, #55-56, were found to react with both anti-rKBG60 and anti-rKBG8.3 sera, only one peptide #29 was mismatched between these two sera. The amino acid sequences of the peptides recognized by the antisera and their locations are presented in Fig.2. It is inferred that a total of 23 peptides were found to be capable of binding to the specific antibodies, and these 23 peptides may represent at least 12 antibody-binding epitopes on the rKBG60.

Although human sera were also used for detection of human IgE antibody-binding epitopes, we failed to detect any binding of the antibodies to the peptides, with allergic human sera diluted in the range of 1:20 to 1:2.

Epitopes defined with human IgE and IgG antibodies

In order to detect human IgG and IgE antibody binding epitopes, 23 synthetic peptides spanning the major part of the rKBG60 were synthesized and examined with the serum pools.

Human IgG binding peptides: Scanning of the overlapping peptides with the serum pool from patients allergic to KBG pollen led to the identification of peptides #6, #9-#12, #17, #21, #23, and #27-#28, which were capable of binding to human IgG antibodies. These ten peptides clearly showed higher binding than the background as determined by ELISA and were therefore inferred as the human IgG binding determinants (Fig.3a). However, it was difficult to evaluate the IgG binding of some peptides, such as

peptides #8, #14, #18, #20 and #25, because their O.D. reading was not high enough to be considered as positives. Scanning of these peptides with the control serum pool revealed that two peptides #10 and #27 had relatively higher binding activities, and the other peptides showed low or no binding (Fig.3b). The two overlapping peptide regions, #9-#12 and #27-#28, were considered to represent at least one epitope, respectively. These results indicated that the detectable ten peptides represented at least 6 epitopes of the rKBG60 involved in human IgG antibody binding. The amino acid sequences and the locations of these peptides are shown in Fig.4.

Human IgE binding 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, #23, and #24-#28 (Fig.5a). These positive peptides were determined by repeated experiments and showed high frequency to react with human IgE antibodies. The control serum pool showed little binding to some peptides, such as peptides #9 and #27, and regarded as non-specific binding (Fig.5b). The results demonstrated that IgE binding epitopes of the rKBG60 located mainly on its C-terminal region (Fig.6) and 10 peptides, representing at least 4 epitopes, were involved in the human IgE antibody binding.

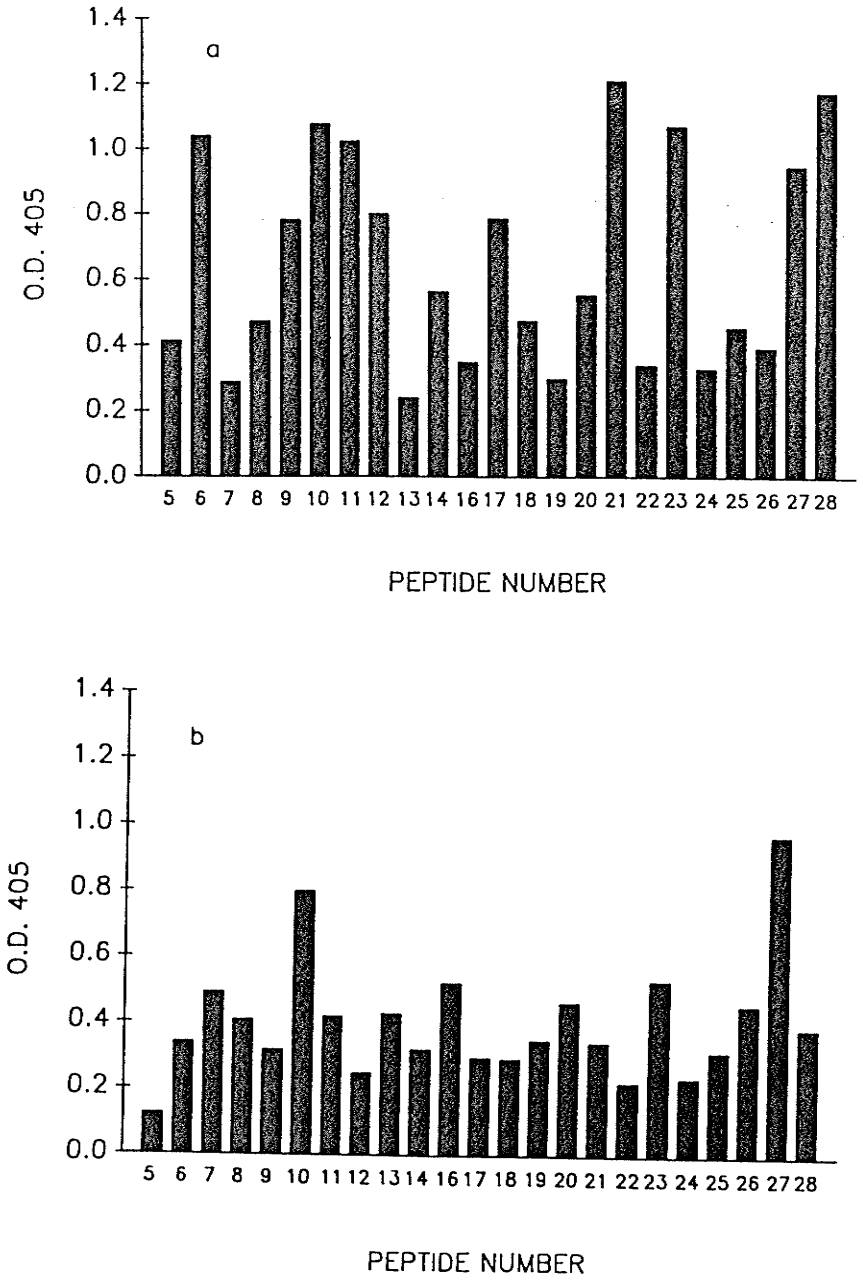


Fig.3 Human IgG binding epitopes defined with the 20 residue peptides. The IgG antibody binding patterns of the serum pools from the KBG allergic individuals(a) and non-KBG allergic individuals(b) were determined with ELISA.

EPITOPE MAPPING WITH HUMAN IgG ANTIBODY

Fig.4 Amino acid sequences and locations of the peptides recognized by the human IgG antibodies. The antibody-binding peptides are underlined.

Both human IgG and IgE antibodies from the atopic serum pool recognized at least ten peptides. Most of these peptides were recognized by both isotype antibodies but with some exceptions. If the ambiguous peptides, such as #8, #14, #18, #20, and #25 were considered as the human IgG positives, then only peptides #24 and #26 were found to be recognized by the human IgE but not by the human IgG antibody from the sera. The peptides #6, #9-#11, and #14, 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.

Synthetic peptides recognized by murine anti-rKBG60 sera

For confirmation of the epitopes defined with the peptides synthesized on the polypropylene pins and comparison of epitopes recognized by human IgE and IgG antibodies with the epitopes defined by the murine antibodies, the free synthetic peptides were also analyzed with murine anti-rKBG60 antisera. The results in Fig.7a clearly shows that peptides #7, #12-#19, #21, #23, and #26-#28 had binding activities to anti-rKBG60 antibodies, whereas the control sera showed no detectable reactivity to the peptides (Fig.7b). The peptides in the region of either the rKBG8.3 or the rKBG60.1 recognized by anti-rKBG60 were also found to be reactive to antibodies raised against the rKBG8.3 and the rKBG60.1, respectively (data not shown). These results demonstrated that there exist at least 13 peptides representing the murine antibody

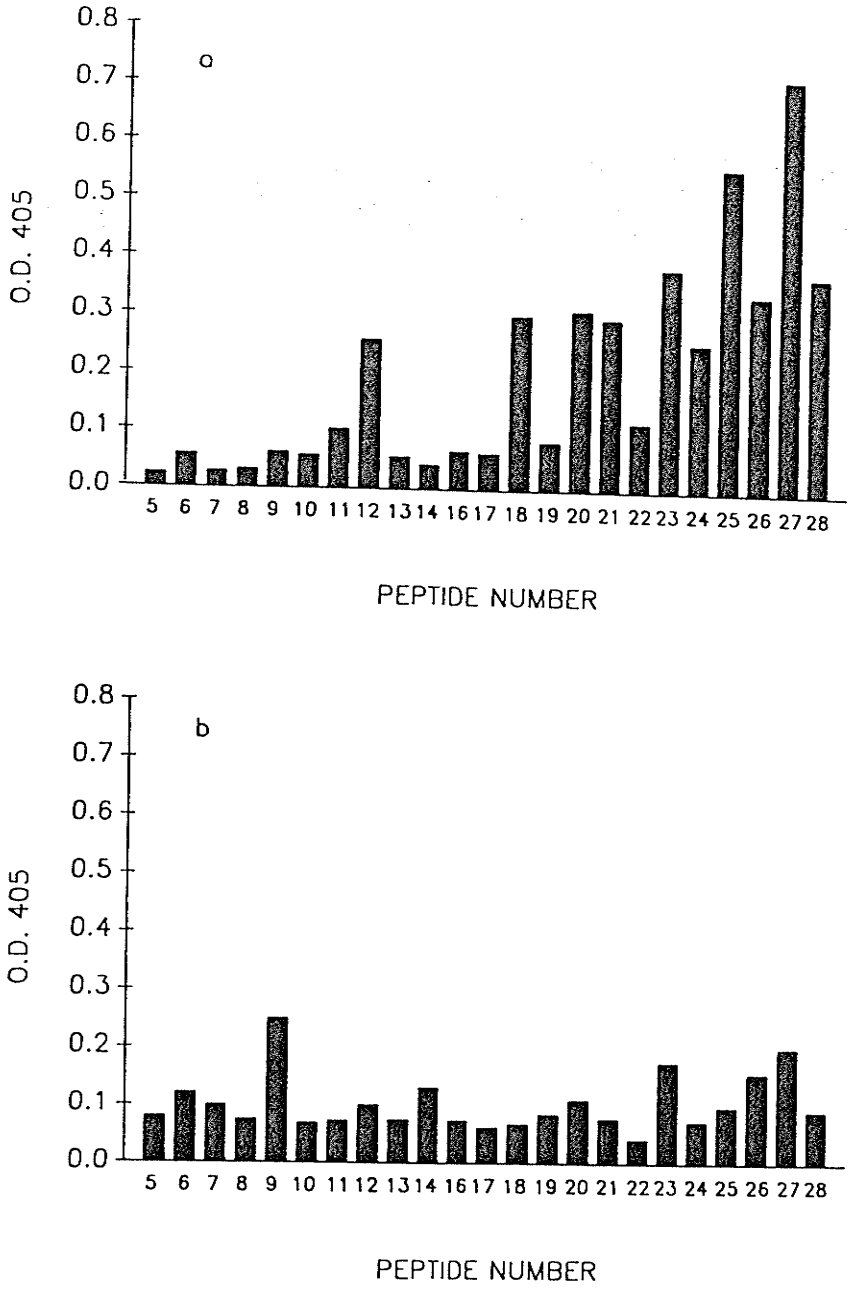


Fig.5 Human IgE binding epitopes defined with the 20 residue peptides. The IgE antibody binding patterns of the serum pools from the KBG allergic individuals(a) and non-KBG allergic individuals(b) were determined using ELISA.

EPITOPE MAPPING WITH HUMAN IgE ANTIBODY

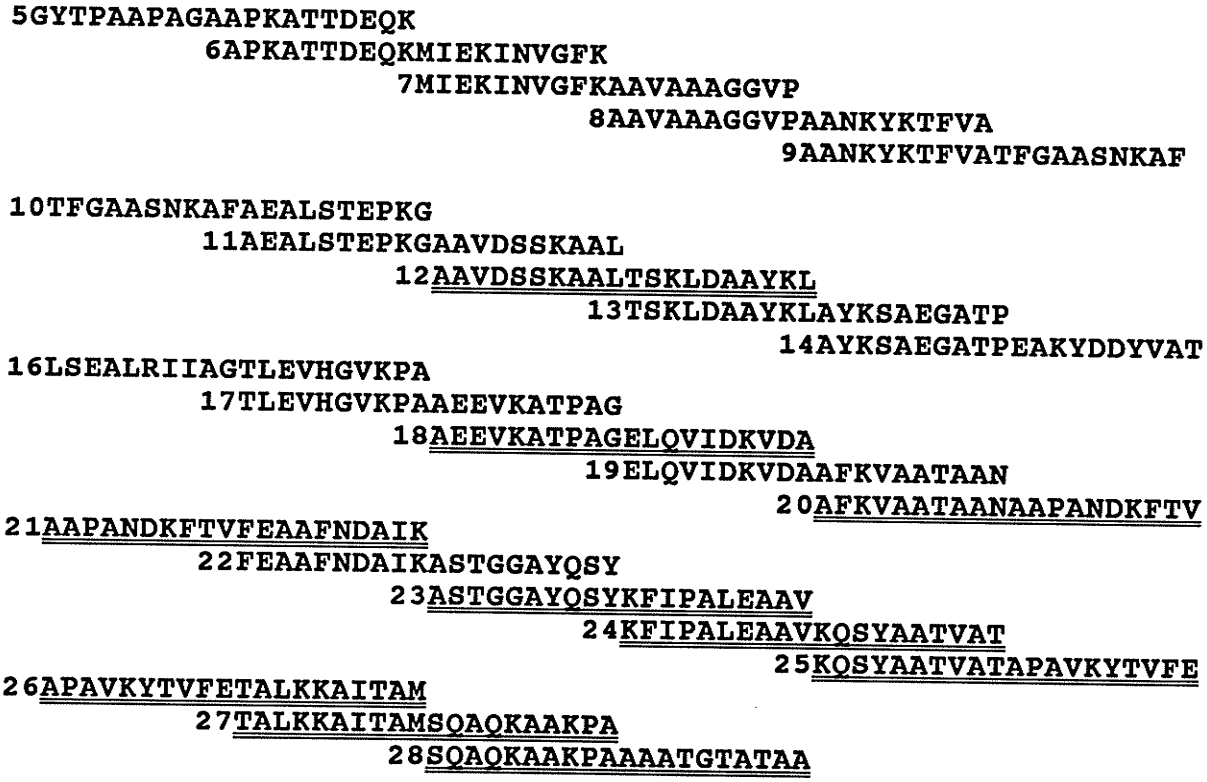


Fig.6 Amino acid sequences and locations of the peptides recognized by the human IgE antibodies. The antibody-binding peptides are underlined.

binding epitopes of the rKBG60, which are scattered mainly around middle and C-terminal region of the protein. The amino acid sequences of these peptides and their locations are presented in Fig.8.

Analysis of the antibody-binding peptides of the rKBG60

Comparison of the positive peptides defined by using the two sets of peptides and murine antibodies: In comparison with the epitopes mapped by using the two different sets of peptides, decapeptides on the polypropylene pins and free 20mer peptides, the sequences of positive peptides were matched in general. Among 23 peptides recognized by the antisera on the pin method, 5 peptides, #2, #3, #4, #55 and #56, were not covered by the 20-residue peptides synthesized with bio-resin method.

Except peptides #13-#14 and #44 of the 18 positive peptides on polypropylene pins recognized by anti-rKBG60 and anti-KBG pollen extract respectively, all the other 15 decapeptides were found to share the sequences with the 20 residue peptides recognized by the antisera. A major difference was observed between the two sets of the peptides, i.e., the amino acid sequence spanned by the 20 residue peptides #26-#28 or by the decapeptides #48-#55, was detected to possess antibody-binding properties only when the 20 residue peptides were used. These results indicated that the epitopes in this region were more conformational in nature and 10 amino acids might be not long enough to form the stable

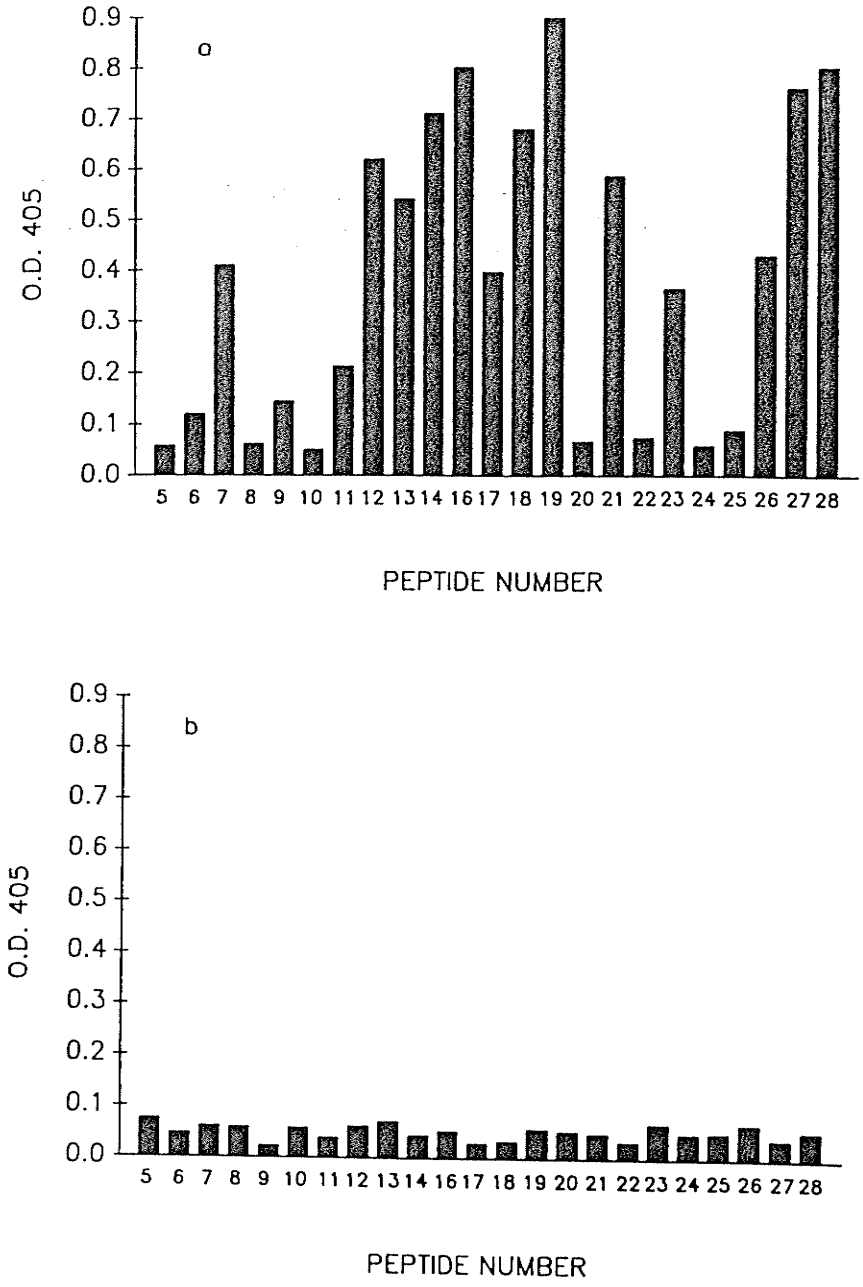


Fig.7 Murine antibody-binding peptides defined with the 20 residue peptides. The murine antibody reactive peptides were defined using anti-rKBG60(a) and negative control sera(b) by ELISA.

EPITOPE MAPPING WITH MURINE ANTISERA

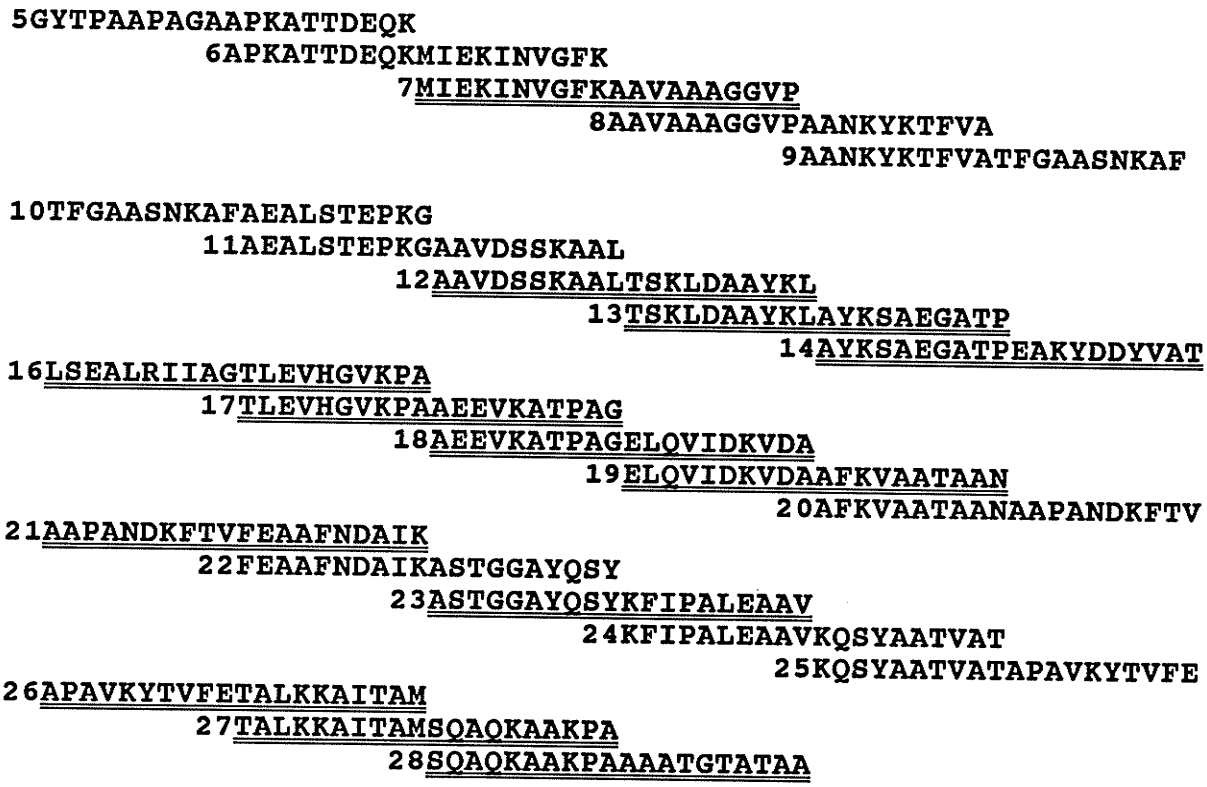


Fig.8 Amino acid sequences and locations of the peptides recognized by the murine antibodies. The antibody-binding peptides are underlined.

antibody binding sites. If this region is considered to contain one epitope, according to the epitope map presented in Fig.2, the conclusion can be drawn that there are at least 13 murine antibody-binding epitopes on the rKBG60. The amino acid sequences involved in the murine antibody binding as determined by using the two sets of the peptides are summarized in Table 2.

The positive 20 residue peptides defined by murine and human antibodies: Murine antisera recognized 13 peptides in total, whereas human IgG and human IgE antibodies each recognized 10 peptides. Although the majority of the peptides recognized by murine sera were also reactive with human IgG and IgE antibodies, especially at C-terminal regions, the 13 murine antibody positives did not cover all the human IgG and IgE positive peptides. The mismatched human IgG- and murine Ig-positive peptides were the peptides #6 versus #7, #9-#12 versus #12-#19. Human IgE antibody positives showed the most restricted pattern. The peptides recognized only by human IgE but not by the murine antisera were #20, #24 and #25. This comparison led to the inference that there are 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(Table 2).

Induction of antibodies with synthetic peptides

To examine the possibility that T cell and B cell epitopes may coincide on the same small peptides, 9 of these peptides, i.e.,

Table 2. Amino acid sequences of the 20 residue peptides reacted with murine anti-rKBG60, human IgG and IgE antibodies

Peptide number	Antibodies			positions on the rKBG60	Amino acid sequence
	mIg	hIgG	hIgE*		
6	-	+	-	29-48	APKATTDEQKMIEKINVGFK#
7	+	-	-	39-58	MIEKINVGFKAAVAAAGGVP
9	-	+	-	59-78	AANKYKTFVATFGAASNKAF
10	-	+	-	69-88	TFGAASNKAFAEALSTEPKG
11	-	+	-	79-98	AEALSTEPKGAAVDSSKAAL
12	+	+	+	89-108	AAVDSSKAALTSKLDAAYKL
13	+	-	-	99-118	TSKLDAAYKLAYKSAEGATP
14	+	-	-	109-128	AYKSAEGATPEAKYDDYVAT
16	+	-	-	129-148	LSEALRIIAGTLEVHGVKPA
17	+	+	-	139-158	TLEVHGVKPAAEEVKATPAG
18	+	-	+	149-168	AEEVKATPAGELQVIDKVDA
19	+	-	-	159-178	ELQVIDKVDAAFKVAATAAN
20	-	-	+	169-188	AFKVAATAANAAPANDKFTV
21	+	+	+	179-198	AAPANDKFTVFEAAFNDAIK
23	+	+	+	199-218	ASTGGAYQSYKFIPALEAAV
24	-	-	+	209-228	KFIPALEAAVKQSYAATVAT
25	-	-	+	219-238	KQSYAATVATAPAVKYTVFE
26	+	-	+	229-248	APAVKYTVFETALKKAITAM
27	+	+	+	239-258	TALKKAITAMSQAQKAAKPA
28	+	+	+	249-268	SQAQKAAKPAAAATGTATAA

* mIg: murine antibody; hIgG: human IgG; hIgE: human IgE.
\# The underlined sequences were also found to be involved in the antibody bindings in the decapeptides on the pins.

peptides #14, #16, #18, #19, #21, #23, #26, #27, and #28, which bound to the murine antibodies, and three peptides, i.e., #5, #22, and #25, which did not bind to the murine antibodies, were used to induce the antibody production in the mice. After second immunization of the mice, the sera were tested and found no detectable specific antibodies were found, indicating the weak immunogenicity of the peptides. The antibodies induced by the peptides were detected only after third immunization. The peptides

which showed no antibody induction after third immunization failed to induce antibody after further immunization. Among the peptides used in immunization, eight induced the allergen-specific antibodies, whereas four peptides including two non-antibody binding peptides, #5 and #22, and two antibody-binding peptides, #14 and #21, induced no detectable antibodies (Fig.9). From the reactivities of the peptide-induced antisera, it was demonstrated that peptides #16, #18, #19, #23, #25, #26, #27 and #28 were immunogenic. The sera from the non-immunized mice used as control showed no binding to the coated allergen.

The anti-peptide sera were also tested for their binding abilities to the peptides in order to detect the possible peptide-induced antibodies, which only recognize the corresponding peptides but not the allergen. These 12 peptides were examined in two experiments, with 6 peptides in each. The results presented in Fig.10 were the binding abilities of the anti-peptide sera to itself with other five coated peptides as negative controls. The first test contained peptides #5, #18, #19, #21, #22, and #23 (Fig.10A). The sera raised against peptides #18, #19, and #23 were found to react with the corresponding coated peptides, whereas the sera against peptides #5, #21, and #22 showed no or little non-specific binding. The sera against peptide #18 bound to the adjacent peptide #19 but not to peptide #18 and other peptides. The other 6 anti-peptide sera against peptides #14, #16, #25, #26, #27, and #28, all showed the binding to the corresponding coated peptides except the anti-peptide #14 (Fig.10b). As the anti-peptide

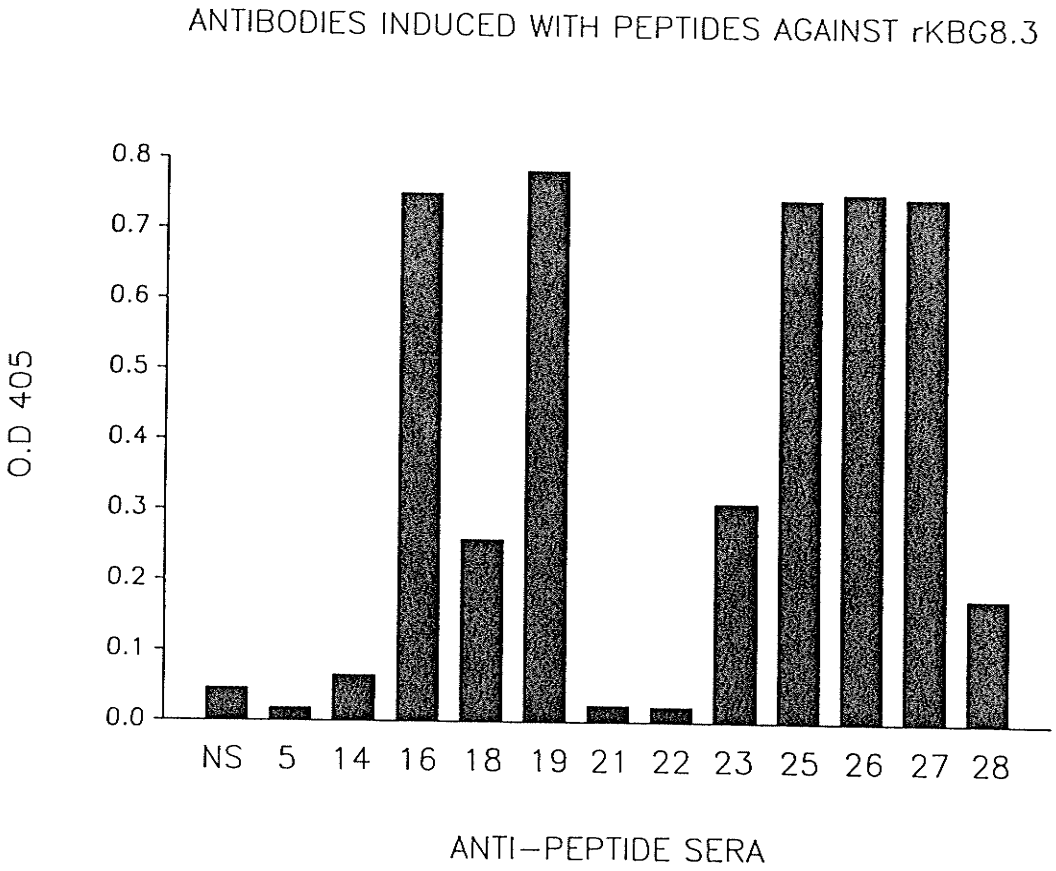


Fig.9 The allergen-specific antibodies induced with the synthetic peptides. The antisera from the mice immunized with the 12 20mer peptides were assayed in ELISA to determine their bindings to the free rKBG8.3. The non-immunized mouse sera were used as negative control.

#18 serum, anti-peptide#28 also showed the binding to the adjacent peptides but not to itself. The antisera against peptides #25-#28 appeared to share some common specificities. Taken together, the results demonstrated that among these 12 peptides eight of them were able to induce the specific antibodies.

DISCUSSION

The present study aimed at delineation of the antibody-binding epitopes of the rKBG60 allergen. Three independent methods were utilized for the synthesis of overlapping peptides: (i) recombinant polypeptides expressed from the cDNA clones, (ii) decapeptides synthesized by the pin method, and (iii) 20 residue peptides produced by an automated peptide synthesizer. Each method has its advantages and disadvantages. The overlapping peptides synthesized on polypropylene pins offer several advantages, including smaller regions of the molecule were evaluated and the procedure to synthesize the peptides is relatively simple in this study. Therefore, it is relatively inexpensive to obtain a large number of peptides. The validity of the epitopes defined on the pins with murine antibodies were confirmed by using the purified peptides which were synthesized by automated procedures and further purified by HPLC. Thus, our definition of the murine antibody-binding epitopes on the rKBG60 were based on the combined results from the peptides synthesized by both pin method and automated procedure.

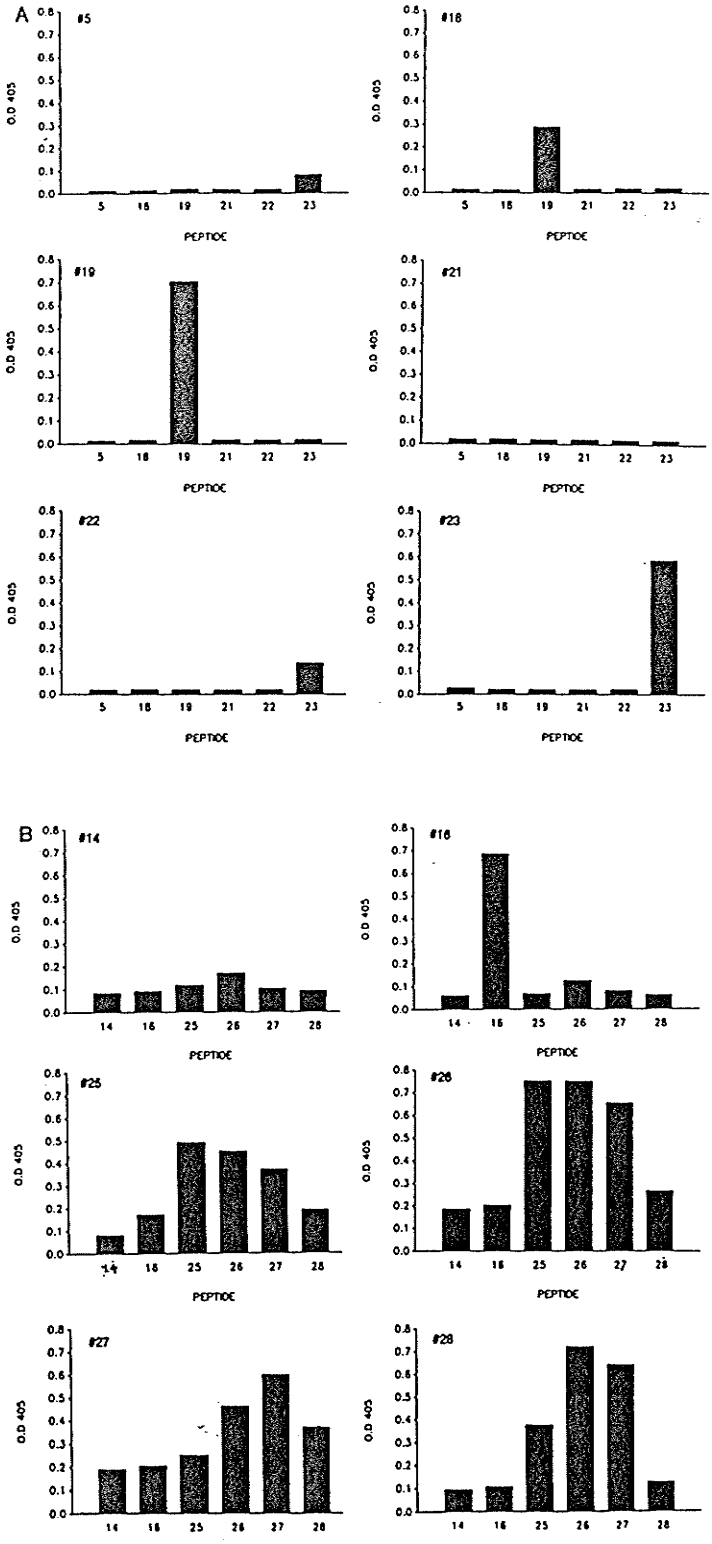


Fig.10 The reactivities of the synthetic peptides with the antibodies induced with the peptides. The peptides used in immunization were coated onto the ELISA plates to determine the binding activities of the anti-peptide sera. Two sets, A and B, of the experiments were performed using six peptides as detecting antigens in each set.

It is to be noted, however, that application of the short synthetic peptides is limited to mapping of sequential epitopes. For instance, the decapeptides synthesized on the pins applied herein would not be useful to identify the solely conformational epitopes nor the epitopes composed with more than ten essential amino acids. Moreover, in keeping with the limitations of the "pin" method of epitope mapping, a lack of binding does not reflect the absolute absence of antibody to this nominal sequence(26). One interesting observation of this study was the region spanned by #26 to #28 of the 20 residue peptides or #46 to #53 of the fixed peptides on the pins. The fixed peptides representing this region could not be recognized by murine antisera, whereas it appeared strongly reactive when the 20 residue peptides were used. The reasons for this discordance are unclear at present. However, it is likely that the epitopes in these peptides requires more than ten amino acids to exhibit the antibody-binding activity. The epitopes requiring certain numbers of amino acids to form topographical contiguous conformational epitopes were reported recently on protein antigens, such as *Der p* I allergen and Sm B/B' autoantigen (27,28).

Different patterns of peptide recognition have been observed in our studies by using different antisera. These results are consistent with similar studies from other laboratories(26,29,30). Thus, different recognition of peptides was seen while using antisera from different animals immunized with the same antigen(26) or from animals immunized with the same antigen but in different

forms(29,30). The discordance in the recognition patterns of the peptides on the pins by three antisera, i.e., anti-KBG, anti-rKBG60 and anti-rKBG8.3, may be due to (i) KBG pollen extract contains a large number of immunogenic protein components(31), therefore, the fact that the titer of antibody to the particular peptide may be too low to be detectable can not be ruled out; (ii) the native KBG pollen proteins, rKBG60 and rKBG8.3 may fold differently such that the peptide(s) in question is(are) not exposed on the surface; in particular, the recombinant allergens are in form of fusion protein with β-galactosidase.

The another limitation of the pin method is that the peptides synthesized on the pins were too low in amount and as such were unsuitable for the definition of the human IgE antibody-binding epitopes(12,15). Our initial attempts to map the IgE binding epitopes by using human sera were also inconclusive. On the other hand, the results of binding studies using the 20mer peptides demonstrated relatively better resolution in terms of epitope mapping with human IgG and IgE antibodies. The findings that most epitopes were recognized by both human IgG and IgE antibodies and IgG antibodies recognized relatively more peptides than IgE antibodies are in general agreement with the report from Thomas et al(27).

The mapping of epitopes utilizing human IgE antibodies confirmed the previous observation that the epitopes on the rKBG60 appear to be distributed mainly on the C-terminal region of the molecule (data presented in Chapter II). The fragment, represented

by the rKBG8.3, spans from the third amino acid of the 20mer peptide #11 to the C-terminus of the molecule. Thus, it covers all the IgE epitopes defined by using the peptides. Although the rKBG60.1 and the rKBG60.2 share the same C-terminus at seventh amino acid of 20mer peptide #19 and cover two IgE binding epitopes, peptides #12 and #18, the differences of the IgE binding activities of these two fragments and the evidence that non-complete inhibition of IgE binding to the full length allergen by the rKBG8.3 (results in Chapter II) indicate there may be some IgE epitopes at the N-terminus of the rKBG60. The reason of inability to detect these IgE epitopes may be due to that the epitopes are on the region represented by the decapeptides #2-#4 on polypropylene pins which are not covered by the 20 residue peptides or the 20 residue peptides may not be long enough for those epitopes to have IgE binding activities.

Unlike the human IgE epitopes, human IgG and murine antibody specific epitopes were also localized around middle region of the rKBG60. The mapping of murine antibody-binding epitopes is consistent with the results of probing the overlapping recombinant polypeptides with murine antibodies (data in Chapter II). It is clear from the comparison of the peptides recognized by human IgG and IgE and murine antibodies that most of the IgE reactive peptides were able to react with human IgG and murine antibodies. Moreover, the few peptides recognized only by human IgE antibodies are always adjacent to the epitopes of murine or human IgG antibodies. These results also provide the rationale for the

inhibition assays used previously with monoclonal antibodies(Mabs) which were useful to localize the IgE epitopes. However, in view of the discordance between peptides recognized by the murine IgG and human IgE antibodies, Mabs may not be useful for precise detection of the structure of allergenic epitopes.

The approach to detect the T cell epitopes in the present study is based on the principle that a short peptide can elicit antibody production only if it possesses three sites recognized by B cell surface Ig, class II MHC molecule and T cell receptor(32-34). Accordingly, the antibody-binding peptides able to induce the allergen-specific antibodies without coupling to carrier macromolecule are considered also to contain T cell epitopes. As reported before that majority of B- and T-cell epitopes were found to locate on the same synthetic peptide(11), our results also demonstrated that the coincidence of B- and T-cell epitopes is a very common phenomenon. Thus, definition of antibody binding epitopes of an antigen also helps further localize the T cell epitopes.

The importance of the T cell epitopes in determination of antibody production has been emphasized by the observations that T cell epitopes from foreign proteins provide help to the B cell epitopes to induce antibodies recognizing the corresponding native proteins when the two kinds of epitopes are linked(35,36). Previously, studies from our laboratory aimed at determining T cell epitopes of *Poa p* IX allergens by T cell proliferation assay revealed that the peptide #25 is T cell epitope(unpublished data).

In this report, this peptide was found to be recognized by human IgE but not by murine antibodies. Whether this peptide was also a murine B cell epitope remained unresolved. Direct immunization of the mice with the peptide induced antibodies, indicating that it is also antigenic to mice.

The observation that some synthetic peptides induce antibodies which only recognize the peptides themselves but not the intact antigens(37) was not observed in our study by using the peptides as coated antigen. However, it can be inferred from our data that the antibodies induced by the peptides #18 and #28 did not or very weakly react with themselves but with adjacent peptides. This possible blockage of the binding sites of the peptides on the ELISA plates suggests the necessity to use the overlapping peptides to confirm the immunogenicity of the peptides. Furthermore, the antibodies induced by the peptides from #25 to #28 were cross-reactive with each other, indicating the common antibody-binding structures conformational in nature in these peptides.

Finally, it should be pointed out from this study that several different strategies have to be used in order to achieve the full profile of antibody-binding epitopes of an antigen. The differences between epitopes recognized by human and murine antibodies, human IgG and IgE antibodies in this report may be due to mainly (i) only one strain mice (b/d haplotype hybrid) used; (ii) limited number of human sera tested. Therefore, further analysis of the epitopes by employing mice of different haplotypes and more individual human sera may lead to different epitope patterns. Furthermore, an

epitope might be able to react with both IgG and IgE antibodies, however, relatively high titer of IgG antibodies could mask the IgE binding to the epitope. Notwithstanding the above limitations, a combination of different strategies of synthetic peptides and different sera appeared to have revealed the majority of the epitopes of the rKBG60 allergen.

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CHAPTER IV

ALLERGENIC AND ANTIGENIC CROSS-REACTIVITIES OF GROUP IX GRASS POLLEN ALLERGENS

ABSTRACT

The allergenic and antigenic cross-reactivities between a recombinant *Poa p* IX allergen, rKBG8.3 and its corresponding proteins of different grass pollens were examined. Immunoblotting of the proteins of thirteen different grass pollens using anti-rKBG8.3 antibodies indicated that *Poa p* IX-like proteins are present in ten other grass pollens, albeit in variable amounts and molecular masses. These proteins ranged in size from 20 to 88 kDa in different grass pollens. The per cent relative binding determined for each grass pollen extract using allergic human sera showed a significant correlation (r=0.891) with that of anti-rKBG8.3 antiserum. Moreover, there was a strong association (r=0.901) between the KBG extract and rKBG8.3 with respect to their inhibition of the binding of human IgE antibodies to allergens in grass pollen extracts. Taken together, these results suggest that the allergenic and antigenic epitopes of the *Poa p* IX-related proteins, in some but not all, grass pollens are similar in structure and specificities. It is concluded that the Group IX allergens constitute a major family of proteins in a variety of different species of grass pollens.

INTRODUCTION

The major drawbacks that undermine the efficacy of immunotherapy in relation to allergies to plant pollens are: (i) the large number of undefined allergenic components to which an individual may be allergic, (ii) lack of knowledge of a given pollen allergen and of the precise allergenic and antigenic relationships among components of various pollens, and (iii) most importantly, the lack of sufficient amounts of pure and standardized preparations of allergenic proteins which could be used for a diagnostic identification of the allergenic components responsible for the sensitivity of a patient. In attempts to demonstrate allergenic cross-reactivity among pollens of different grasses *in vitro* techniques, such as the radioallergosorbent test (RAST) and immunodiffusion analysis (1-5), and the *in vivo* skin prick test (6-8) have been employed. Recently, murine monoclonal antibodies (Mabs) to purified allergenic components have been used to investigate cross-reactivities among grass pollens (9-12), whereby each Mab is directed against a unique epitope. However, this approach has two potential pitfalls: (i) the single epitope recognized by the Mab may not be representative of relative allergenic potency of the protein(s), and (ii) it may be difficult to identify Mabs to minor component(s) which may, nevertheless, constitute important allergens in patients.

As stated earlier, the molecular cloning and sequencing of a

number of cDNA clones led to identification of a new group of allergenic proteins. The protein equivalents of these recombinant allergens were identified using an antiserum raised in mice to the fusion protein which consisted of a truncated β-galactosidase fused to a polypeptide encoded by a cDNA clone, KBG8.3. This recombinant polypeptide was shown to contain a number of major B cell and T cell epitopes. In this study, the presence of *Poa p* IX-related proteins in ten other different grass pollens was established by using the murine anti-rKBG8.3 serum and the pooled sera of KBG-allergic individuals.

MATERIALS AND METHODS

Preparation of the recombinant allergenic polypeptides

The rKBG8.3 allergenic polypeptide was synthesized in *E. coli* and purified according to the procedures described in Chapter II.

Murine antibodies

BDF1 mice, female 6-8 weeks old, (Jackson Laboratories, Bar Harbor, MI), were immunized with the rKBG8.3, KBG pollen extract and β-galactosidase(BRL, CA) in dextran sulfate (Sigma, MO) as adjuvant. Three mice in each group received the same antigen, with a mouse primed with 5 μg of either the recombinant KBG8.3 or β-galactosidase protein and 20μg of the KBG pollen extract in 2.5mg dextran sulfate. The mice were boosted 23 days later with the same

dose of the proteins and 1 mg of dextran sulfate; 7 days later they were bled and their sera were stored at -20^{0}C.

Preparation of grass pollen proteins:

The proteins of 13 different grass pollens (Kentucky Blue, Bermuda, Canary, Orchard, Redtop, Smooth brome, Tall oat, Perennial rye, Timothy, Colonial bent, Quack, Johnson, and Reed canary, Hollister-Stier Laboratory, Mississauga, ON) were extracted with distilled water at 4^{0}C overnight (18). The protein concentration of each extract was determined by Lowry's method (19).

SDS-PAGE and Immunodetection:

SDS-PAGE was performed on a vertical slab gel apparatus using a 15% polyacrylamide gel to analyze 13 grass pollen extracts according to the method of Laemmli (20). In each lane, 20μg of grass pollen extract was loaded, followed by electrophoresis for separation of the proteins. The molecular weights of the components were estimated with reference to the mobilities of prestained protein standards (Bio-Rad Laboratories, Mississauga, ON). The separated proteins were either stained with Coomassie Blue or electroblotted onto nitrocellulose membrane. The membrane was then probed with a 4000-fold diluted murine antiserum raised against the rKBG8.3 polypeptide, followed by incubation with a conjugate of goat antibodies to murine immunoglobulins and alkaline phosphatase (Zymed, CA) as described before(21).

Enzyme linked immunoassay (ELISA):

The ELISA used for the determination of the specificities of antiserum to the rKBG8.3 and to KBG pollen extract is described below. One μg of KBG extract in 100 μl of carbonate/bicarbonate buffer, pH 9.6, was coated onto each well of a Nunc-immuno plate overnight at room temperature. After washing three times with PBS-tween buffer, the plate was blocked with 1% gelatin in PBS for 2 hours at 37^0C followed by addition of serially diluted murine antisera specifically raised against either rKBG8.3, or β-galatosidase, or KBG extract. Normal mouse serum was used as control. The bound antibodies were detected by adding the alkaline phosphatase-conjugated goat anti-mouse IgG(Zymed) at 1/2,000 dilution and the enzyme substrate *p*-nitrophenyl phosphate in alkaline phosphatase (AP) buffer. The ability of the antisera raised against the rKBG8.3 to react with 13 different grass pollen extracts was examined essentially as described above except that the plates were coated in turn with each different pollen extract, and the antisera to the rKBG8.3 were used at a dilution of 1:2,000.

The allergenic cross-reactivities between the different grass pollens were evaluated by ELISA utilizing the serum pool from the individuals allergic to grass pollens described in Chapter II. Briefly, 1 μg protein in 100 μl of carbonate/bicarbonate buffer of each of the 13 grass pollen extracts was coated onto Nunc-immuno-plates by overnight incubation at room temperature. The plates were then washed three times with PBS-Tween buffer, blocked with 1% gelatin in PBS, and then incubated with the 1 in 10 diluted serum

pool overnight at room temperature. Bound IgE antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgE(TAGO, CA) and its substrate *p*-nitrophenyl phosphate. The absorbance was read at 405 nM using a microplate reader(Dynatech). The reactivity of the KBG allergen-specific antibodies with other grass pollen extracts was calculated according to a previously described method(9) as:

% relative binding = binding to heterologous pollen extract/binding to KBG extract X 100.

Immunoinhibition:

Antigenic cross-reactivity of the rKBG8.3 with different grass pollen allergens was analyzed by competition assay. A Nunc-immuno plate was coated with pollen proteins of each grass pollen, each well containing 1 μg of protein in 100 μl of carbonate/bicarbonate buffer, at room temperature overnight. After washing and blocking with 1% gelatin in PBS, three of the 6 wells were incubated with the 1 in 40 diluted human serum pool in absence of inhibitor; the other three wells were incubated with the same serum pool but in presence of KBG pollen protein or the serum absorbed with the rKBG8.3.

KBG pollen protein (1 mg/ml) was used in the solution phase for the inhibition assay, whereas for the inhibition assay involving the rKBG8.3 the serum was first absorbed with the rKBG8.3 (0.5 mg/ml), centrifuged at 10,000 rpm for 10 minutes to remove the

insoluble rKBG8.3-antibody complexes and diluted 40-fold prior to its use in ELISA. These assays were performed in triplicate. After overnight incubation at room temperature, the plate was washed three times. The IgE bound was detected with alkaline phosphatase-conjugated goat anti-human IgE(TAGO) and its substrate *p*-nitrophenyl phosphate. The absorbance was read at 405 nM. The results were expressed as a percentage of inhibition, calculated as follows:

[1-(O.D in presence of inhibitor/O.D in absence of inhibitor)]x100.

RESULTS

Specificities of the murine antisera

The specificity of the murine anti-rKBG8.3 antibodies was analyzed by both SDS-PAGE immunoblotting assay and ELISA using proteins of KBG, short ragweed pollen, and β-galactosidase as antigens. Since the rKBG8.3 allergen synthesized by *E. coli* was associated with a truncated β-galactosidase protein, the antibodies raised against this fusion protein were directed not only to the recombinant KBG polypeptide but also to the above β-galactosidase protein. The antibodies, therefore, recognized KBG proteins and β-galactosidase but not short ragweed proteins (data not shown). The possibility of cross-reactivity between KBG proteins and β-galactosidase was examined by an ELISA assay as shown in Fig.1. The

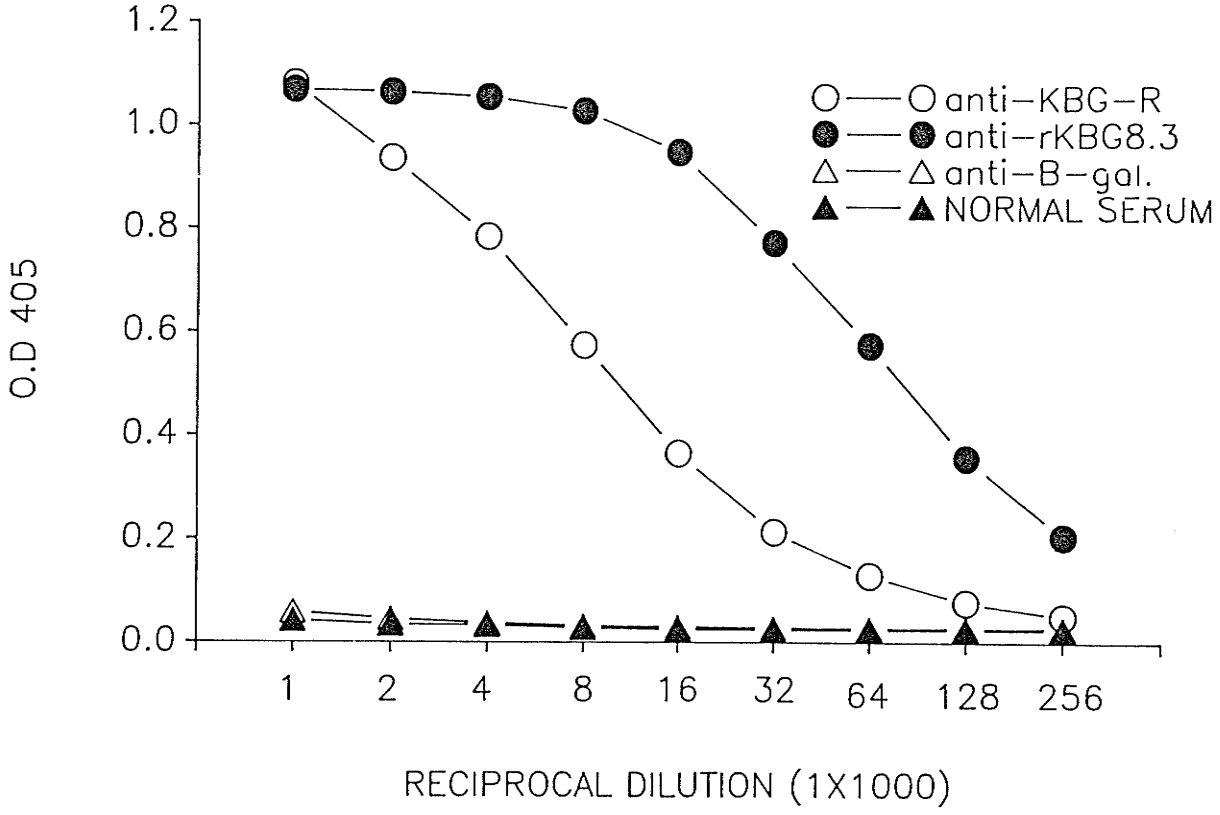

Fig. 1. Non-overlapping specificity between antibodies to KBG extract and β-galactosidase demonstrated by ELISA. 1 μg of KBG extract was used for coating each well. Four murine antisera with serial dilutions were tested their reactivity with KBG extract. Antibodies to both KBG extract and rKBG 8.3 strongly reacted with the coating antigen, but antibodies to β-galactosidase reacted to KBG antigens as low as normal murine sera.

antibodies raised against both KBG pollen extract and the rKBG8.3 reacted with the solid-phase KBG proteins, whereas there was no detectable reaction with β-galactosidase antibodies as well as the normal mouse serum pool, indicating that there is no cross-reactivity between KBG pollen proteins and β-galactosidase.

Identification of the rKBG8.3 cross-reactive components in 13 grass pollen extracts

An electrophotogram of the SDS-PAGE gel which was stained with Coomassie Blue is shown in Fig. 2A. One 28 kDa component and a very faint 32kDa component were detected in protein extract of KBG pollen as well as protein extracts of 12 other grass pollens. Anti-rKBG8.3 serum was employed as a probe to examine its ability to recognize the cross-reactive components in the 13 grass pollen protein extracts (Fig. 2B). Three components with molecular masses of 28, 32 and 34 kDa were detected in KBG pollen extract. Except for the Bermuda and Johnson grass pollen extracts, all other 11 grass pollen extracts possessed components recognized by the anti-KBG8.3 serum. Moreover, 7 out of 11 grass pollens examined possessed multiple components which reacted with the above antiserum. Most of the components ranged in molecular mass from 20 to 38 kDa. In addition, a component with molecular mass of about 88 kDa was detected in the Smooth brome and Canary extracts (Table 1). These results demonstrated that components cross-reactive with the rKBG8.3 exist in at least ten other grass pollens.

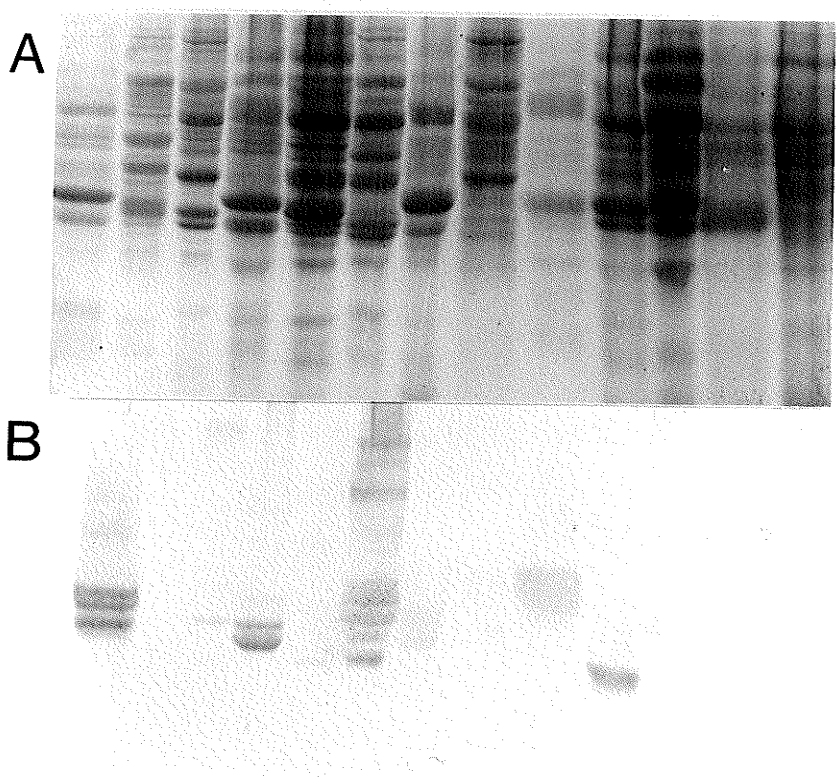

Fig. 2. SDS-PAGE analysis and immunodetection of 13 grass pollen extracts. (A) Protein extracts were electrophoresed on 15% polyacrylamide gel under reduced conditions. The bands were visualized by staining with Coomassie Brilliant Blue. The lanes containing protein extracts from different grass pollens are marked with letters as: A. Kentucky Blue, B. Bermuda, C. Canary, D. Orchard, E. Redtop, F. Smooth brome, G. Tall oak, H. Rye, I. Timothy, J. Colonial, K. Quack, L. Johnson, M. Reed canary. (B) The proteins were separated on SDS-PAGE gel and electroblotted onto nitrocellulose membrane. The membrane was probed with anti-rKBG8.3 antibodies, followed by incubation with enzyme-conjugated second antibody and its substrate.

Table 1 Summary of Components Recognized by anti-rKBG8.3 Antiserum in Different Grass Pollens

CODE	GRASS POLLEN	MOLECULAR SIZES (kDa)
A	KENTUCKY BLUEGRASS	28.0[1], 29.0, 32.0, 34.0
B	BERMUDA GRASS	ND[2]
C	CANARY GRASS	29.4[1], 35.1[1], 88[1]
D	ORCHARD GRASS	24.2, 28.6, 32.9[1]
E	REDTOP GRASS	22.0[1]
F	SMOOTH GRASS	SEVERAL BANDS ABOVE 22.0
G	TALLGRASS	27-32(SEVERAL BANDS)
H	RYEGRASS	28.0[1], 36[1]
I	TIMOTHY GRASS	31-38 (SEVERAL BANDS)
J	COLONIAL GRASS	19.8, 28
K	QUACK GRASS	28.6
L	JOHNSON GRASS	ND[2]
M	REED CANARYGRASS	27.7[1]

[1]Bands of faint color reaction.

[2]ND = not detectable

Reactivity of anti-rKBG8.3 with soluble components of the grass pollen extracts

To rule out the possibility that some antigens may bind poorly to nitrocellulose membrane and therefore escape detection in the western blotting assay, we performed a cross-inhibition experiment using ELISA. Four grass pollen extracts were chosen to inhibit the binding of the rKBG8.3 antibodies to solid-phase KBG proteins (Figure 3). Pollen proteins of Bermuda and Johnson grasses which lacked reactivity with the anti-rKBG8.3 sera in western blotting had no inhibiting effect. Of the two grass pollen extracts which had exhibited the highest reactivity with the antiserum raised against the rKGB8.3, Timothy grass pollen extract inhibited up to 80% of the antibody binding to the solid-phase of KBG extract. However, the inhibition was lower than that seen using KBG protein extract itself. It is inferred from these studies that the proteins recognized by the anti-rKBG8.3 are quantitatively and qualitatively different in various grass pollens.

Relative bindings of different grass pollen extracts with the antisera

The cross-reactivities among the grass pollen components were expressed as relative bindings using KBG pollen proteins as standard and the results are shown in Fig. 4 A and B. The pool of sera of patients allergic to KBG pollen, which were selected on the basis of their reactivity with KBG pollen extract, contained a variety of specificities of IgE antibodies to the other

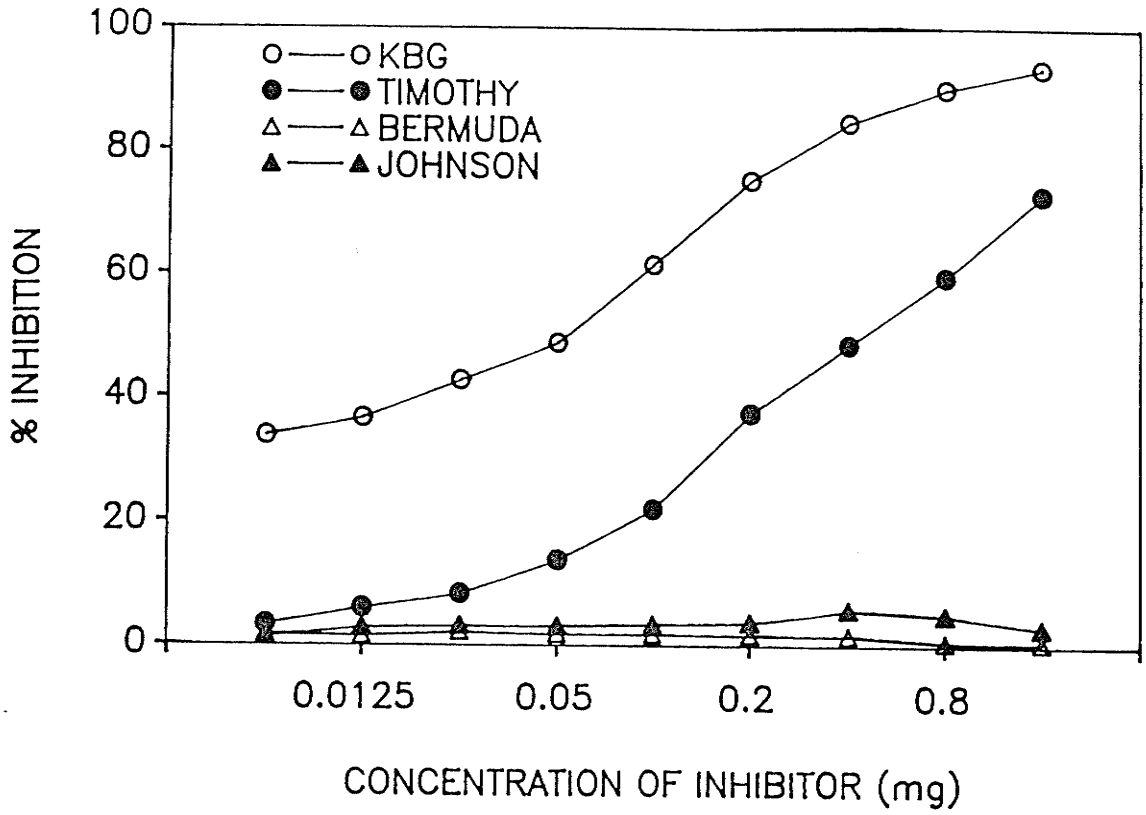

Fig. 3. Cross-inhibition of anti-rKBG8.3 antibodies to KBG extract. Serial concentration of four grass pollen extracts in soluble phase was employed to determine their inhibiting effect on the binding of anti-rKBG8.3 sera to KBG extract in ELISA assay. KBG extract was coated (1 μg per well) onto ELISA plate. Each point represents the mean of three experimental values.

12 grass pollens (Fig. 4A). On the other hand, mouse anti-rKBG8.3 serum showed relatively restricted specificity in its reaction with different grass pollen extracts (Fig. 4B). Thus, the pollen extracts of Orchard, Timothy, Canary, Smooth brome and Tall oak grasses showed higher reactivity than Quack, Rye, Colonial bent and Redtop grasses, whereas the extracts of Bermuda and Johnson grass pollens exhibited no reactivity whatsoever. Furthermore, the relative binding determined for each grass using allergic human sera showed a significant correlation ($r=0.891$) with that determined by using anti-rKBG8.3 antibodies. These results indicate that *Poa p* IX cross-reactive components in different grass pollen extracts vary in their specificities.

Immunoinhibition of the human IgE binding to different grass pollen extracts

The inhibition assay was employed to assess the relative capacities of the KBG extract and the rKBG8.3 to inhibit the binding of human IgE to different grass pollens. The results presented in Fig.5 A demonstrate that KBG extract blocked most IgE binding to Canary, Orchard, Smooth brome, Tall oat, Rye and Timothy; relatively low inhibition of binding to Quack, Colonial and Redtop; with little or no inhibition to Johnson and Bermuda grass pollen extracts. The inhibition profile of the rKBG8.3 had a similar pattern as the KBG extract (Fig. 5 B). Moreover, there was a significant correlation ($r=0.901$) between the KBG extract and the rKBG8.3 with respect to their inhibition of the binding of

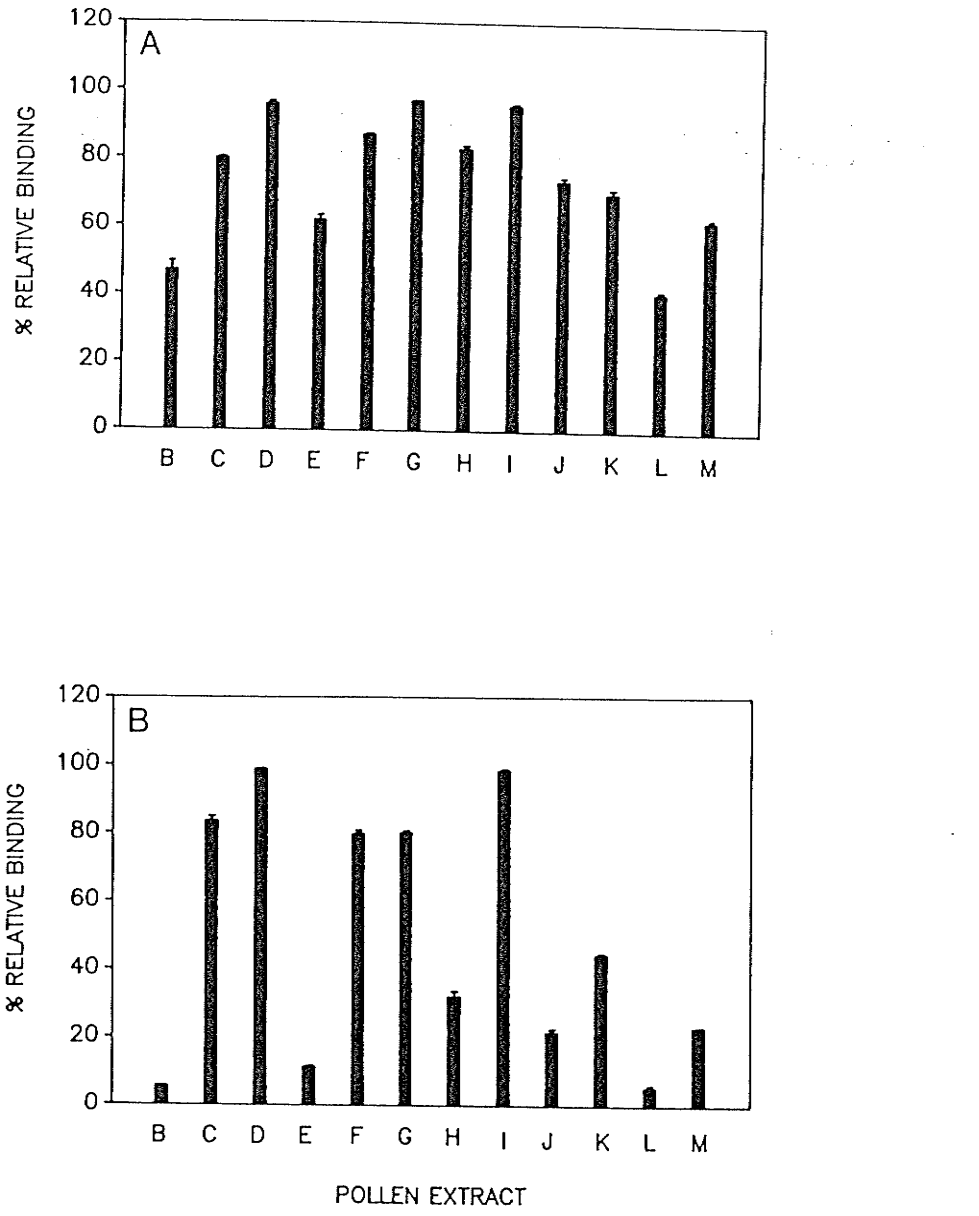

Fig. 4. Binding activities of a highly KBG-allergic human serum pool (a) and anti-rKBG8.3 antibodies (b). Similarities of allergenicity and antigenicity between KBG pollen and the other 12 grass pollens were measured as relative bindings, in which OD values of each grass pollen was compared with that of KBG extract in ELISA assay. The grass pollens in both panels are marked as in Fig. 1.

human IgE antibodies to allergens in the different grass pollen extracts. These results confirm the findings of relative binding studies and suggest that purified allergens can be readily employed in studies of allergenic and antigenic cross-reactivities.

DISCUSSION

Cloning and expression of allergens with recombinant DNA techniques have greatly facilitated the identification, characterization and analysis of their epitopes. In the present study, we have shown the application of recombinant allergens to the study of cross-reactivities among grass pollen components by employing (i) the purified recombinant allergen rKBG8.3 and allergic human sera for the analysis of cross-reactivities among allergenic components, and (ii) a murine monospecific polyclonal antiserum to the above recombinant allergen for the detection of cross-reactive antigenic components.

Previously, several investigators have employed monoclonal antibodies and demonstrated that cross-reactive grass pollen group I allergens, which are similar in molecular weight, exist among different grass pollens (11,12,17). By contrast, immunodetection of allergens in eleven different grass pollens with antibodies to the rKBG8.3 revealed that the cross-reactive allergens ranged in molecular mass from 20 to 88 kDa, although in most of the grasses, the components were in range of 30-35kDa. On the other hand,

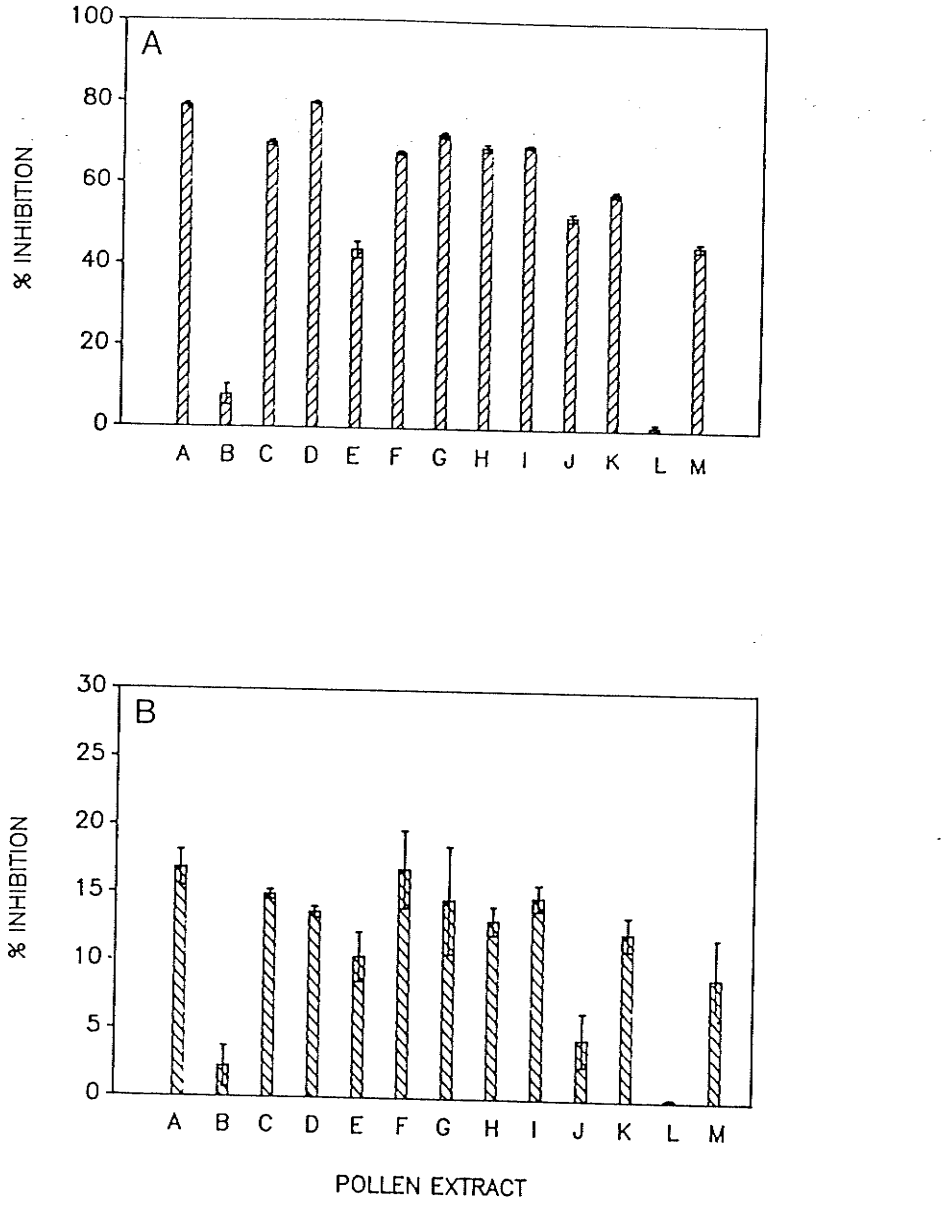

Fig. 5. Immunoinhibition of human IgE binding. The bindings of human IgE to 13 grass pollens marked as in Fig. 1 were inhibited with both KBG extract (a) and purified rKBG8.3 polypeptide (b). The former was used in the solution phase to compete the IgE with the fixed antigens, and the latter was first used to directly absorb the IgE from the serum. Both profiles were obtained by ELISA assay.

Bermuda and Johnson grass pollens possess no detectable the *Poa p* IX components. These results are in agreement with previous findings (1,3) which have indicated that these latter two grasses appear to possess allergenic molecules distinct from other grasses.

The observation that the antibodies to the rKBG8.3 recognized several components in some of the grass pollen extracts is consistent with our previous report that *Poa p* IX was comprised of a group of at least three isoallergenic proteins(18). The different degrees to which the individual components were recognized suggests that the density of epitopes may differ in the individual components or the amount of these components may vary from one grass pollen to another. Furthermore, it appeared that diversity of epitopes in the molecules played a major role in the degree of staining on the nitrocellulose membrane since the 34kDa component in KBG pollen showed strong reaction even if the amount of the protein in the preparation was extremely low. This was also supported by the results of cross inhibition since the highly reactive Timothy extract could not totally abolish the reaction of the anti-rKBG8.3 sera to KBG extract.

The relationship of 12 different grass pollens to KBG in terms of allergenicity and antigenicity was analyzed with relative binding to an allergic human serum pool and the anti-rKBG8.3 antibodies. Clearly, the serum pool contained IgE antibodies to all grass pollens examined. This observation is consistent with our previous finding of a strong positive correlation in levels of IgE Abs corresponding to the *Poa p IX* allergenic peptide and a

mixture of ten grass pollen extracts (19). However the reason for this reactivity, i.e., whether the binding results from IgE antibodies produced as a consequence of contact of human to all grass pollens or due to cross-reactivity is unknown. Moreover, the basis for the observed differences in reactivity of 12 grass pollens to the rKBG8.3 antibodies are unclear. These may be attributed to the geographical distribution of these grasses, or to the similarities in the structures of their allergenic proteins. It is to be noted that the antigenic relationships of these proteins in different grasses did not necessarily agree with their relationships established on the basis of taxonomic classification. For example, timothy and redtop grasses both belong to tribe Agrostideae, but timothy exhibited a higher reactivity with the rKBG8.3 than redtop. Similarly, although Reed canary, Ryegrass and KBG belong to the same tribe Festuceae, Reed canary and Ryegrass showed weaker reactivity with the anti-rKBG8.3 antibodies. This suggests that the taxonomic relationships among grasses does not provide a reliable marker for the presence of individual allergenic proteins and that for each newly cloned allergens the distribution of its epitopes have to be confirmed among other grass components by similar studies as reported here.

In conclusion, this study has clearly pointed out that allergenic and antigenic cross-reactivities widely exist in grass pollens with *Poa p IX* proteins. These results in conjunction with our previous studies of the allergenic properties of the recombinant *Poa p IX* allergens (19) indicate that the Group IX

proteins constitute a major group of allergenic proteins of several grass pollens. Furthermore, the correlations of reactivities of grass pollen extracts with human allergic serum versus anti-rKBG8.3 antiserum suggest that, in general, the epitopes may bind to both IgE and IgG antibodies. However, it is to be pointed out that whether an individual epitope would preferentially bind or recognize the IgE and/or IgG antibodies remains unclear.

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CHAPTER V

INDUCTION OF IgE ANTIBODIES IN MICE WITH RECOMBINANT GRASS POLLEN ALLERGENS

ABSTRACT

In this study, the recombinant *Poa p* IX allergens were examined for their *in vivo* allergenicity and antigenicity. Immunization of BDF1 mice with a fusion protein comprising β-galactosidase and the rKBG8.3 allergen induced high titers of both IgG and IgE antibodies. By contrast, rKBG60.2, which represents an N-terminal fragment of rKBG8.3 induced only IgG antibodies. The IgE antibody titer specific to KBG was significantly higher than that to β-galactosidase. Moreover, KBG specific IgE antibodies showed no apparent decrease in their titers until 60 days after immunization, whereas the β-galactosidase specific IgE antibodies disappeared after 40 days. The antibodies induced with the rKBG8.3 in mice were capable of inhibiting the binding of human IgE antibodies to KBG pollen allergens, which indicated that the rKBG8.3 specific murine antibodies recognized the epitopes similar to the epitopes recognized by human IgE antibodies. Analysis of allergenic cross-reactivities of the 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 the components from other grass pollens. These results suggest that the mice may serve as a model for the manipulation of the induction of and the on-going of IgE responses to the recombinant allergens or their chemically modified derivatives.

INTRODUCTION

Molecular cloning of allergens has advanced our knowledge of (i) the primary structure of the respective allergen molecules(1-6), (ii) the possible biological functions of some of these allergens(1,5), and (iii) the antigenic cross-reactivities among groups of aeroallergenic proteins(3,7,8). However, the progress on the immunological characterization of the recombinant allergens or their fragments have been slow. Most studies in this respect 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(9) or of human peripheral blood mononuclear cells or lines(10). To our knowledge, the analysis of *in vivo* immune responses to recombinant allergens have been limited.

On the basis of the *in vitro* studies, it is conceivable that the recombinant allergens or allergen 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 are, therefore, critically important. The primary interest of this study

was to develop an experimental model for study of the allergenicity of the recombinant allergens. We present evidence here on the IgE and IgG antibody responses *in vivo* of mice to one of the recombinant allergens encoded by cDNA KBG8.3, which represents the common C-terminal region of the *Poa p* IX isoallergens, and encompasses the previously described rKBG7.2(3,9,11).

MATERIALS AND METHODS

Preparation of the recombinant KBG8.3 and KBG60.2 polypeptides

One of the IgE-binding clones viz. KBG8.3 and its N-terminal fragment KBG60.3 were synthesized in *E. coli* strain JM105 with the aid of the expression vector pWR590.1(12). The recombinant polypeptides encoded by the above clones were isolated and purified as described in Chapter II and stored at -20^{0}C.

Grass pollen extracts

The pollen protein extracts were prepared according to the method as described before(13). 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 units; Spectrum Medical Industries, Inc., Los Angeles, CA). The protein concentration was determined using

Lowry's method(14).

Immunization of mice with the recombinant allergens

To produce murine antisera to the rKBG8.3, female B6D2F1 mice (Jackson Laboratories, Bar Harbar, MI), 6-8 weeks old, were immunized with the rKBG8.3 or rKBG60.2 polypeptide dextran sulfate as described in Chapter II. Murine antibodies to KBG protein extract and β-galactosidase (BRL, CA) were also produced using the same method, except that 20μg KBG protein was used for the immunization.

ELISA

KBG- and β-galactosidase-specific IgG antibodies were determined by ELISA. Microtiter plates (Nunc, Denmark) were coated with 1 μg KBG pollen extract or 0.1 μg β-galactosidase for each well, respectively, in 100 μl bicarbonate buffer (0.05 M, pH 9.6) overnight at room temperature. After three washes with PBS-tween (PBST) buffer and saturation of the plates with 1% gelatin in PBS buffer, 100 μl of serially diluted sera were added into wells. After two hour 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 2 hr at room temperature, washed three times and then added the substrate for the color reaction(7). The results were expressed as ELISA titers, the ELISA was considered positive at O.D. values which were three-fold higher than that of

the normal mouse serum pool 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 eleven individuals allergic to Kentucky Bluegrass pollen(9) was used as the source of specific IgE antibodies. Serially diluted antiserum to the rKBG8.3 were added into microplate wells precoated with either 1 μg KBG pollen extract or 0.1 μg rKBG60 and then blocked with gelatin under conditions described above. After two hour incubation at room temperature, the murine serum was discarded and 100 μl 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 ϵ chain conjugated with alkaline phosphatase (TAGO, CA). Direct absorption of the human sera with the rKBG8.3 was used as control(8).

Passive Cutaneous Anaphylaxis (PCA)

Antigen-specific IgE antibodies were determined as PCA titers. Hooded rats were sensitized with 50 μl of serially diluted mouse sera injected intradermally on the backs. 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 β-galactosidase (BRL, Mississauga, ON), in 1 ml PBS containing 0.5% Evan's blue. The PCA titer was the highest dilution giving a clearly visible skin reaction (>5mm in diameter).

Cross absorption of antiserum to the rKBG8.3 and KBG extract

with their respective antigens was performed on solid phase using a method described before(7). Briefly, the proteins, 10 μg of the rKBG8.3 or 100 μg of KBG extract, were dotted on nitrocellulose and the membrane was air-dried for 10 minutes, followed by incubation in PBS buffer containing 2% BSA for 1 hour at room temperature. The membrane was then transferred into wells containing the 1 in 20 diluted antiserum. After 2 hour incubation at room temperature, the antiserum was collected and serially diluted for PCA assay. β-galactosidase and bacterial proteins were used as controls.

RESULTS

Immune responses to fusion proteins

Immunization of mice with the fusion proteins (FP) containing either the rKBG8.3 or the rKBG60.2 led to induction of antibodies to both the corresponding KBG pollen allergens (KBG) and to β-galactosidase. The FP containing the rKBG8.3 induced high titers of IgG antibodies to both the allergenic peptide and β-galactosidase, and high titers of IgE antibodies to KBG but not to β-galactosidase. The FP comprising the smaller KBG peptide, the rKBG60.2, induced high titer of IgG antibodies to β-galactosidase, but the KBG-specific IgG titer was low. Moreover, IgE antibodies to either KBG or β-galactosidase were not detectable (Table 1). No decrease in the titer of IgG antibodies specific to both KBG and β-

galactosidase was seen up to 3 months after immunization contrary to the IgE titers described below.

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

Anti-sera	KBG (titers)		β-gal. (titers)	
	IgG	IgE	IgG	IgE
rKBG8.3	$512x10^2$	$12.8x10^2$	$512x10^2$	20
rKBG60.2	$4x10^2$	Nil	$128x10^2$	Nil

The possibility of cross-reactivity between KBG and β-galactosidase in PCA assay of IgE detection was ruled out by employing antibodies to KBG and β-galactosidase (Fig. 1). Rats injected with the antiserum to FP responded to challenge of KBG protein extract and β-galactosidase, although the titers for β-galactosidase were very low (slightly above the background). Antiserum to KBG pollen extract or to β-galactosidase reacted with the corresponding antigens, respectively, and exhibited titers similar to that of the antiserum to FP. These results indicate that there is no cross-reactivity between KBG allergens and β-galactosidase, and suggest that the component antigens of FP differ in their ability to elicit IgE responses.

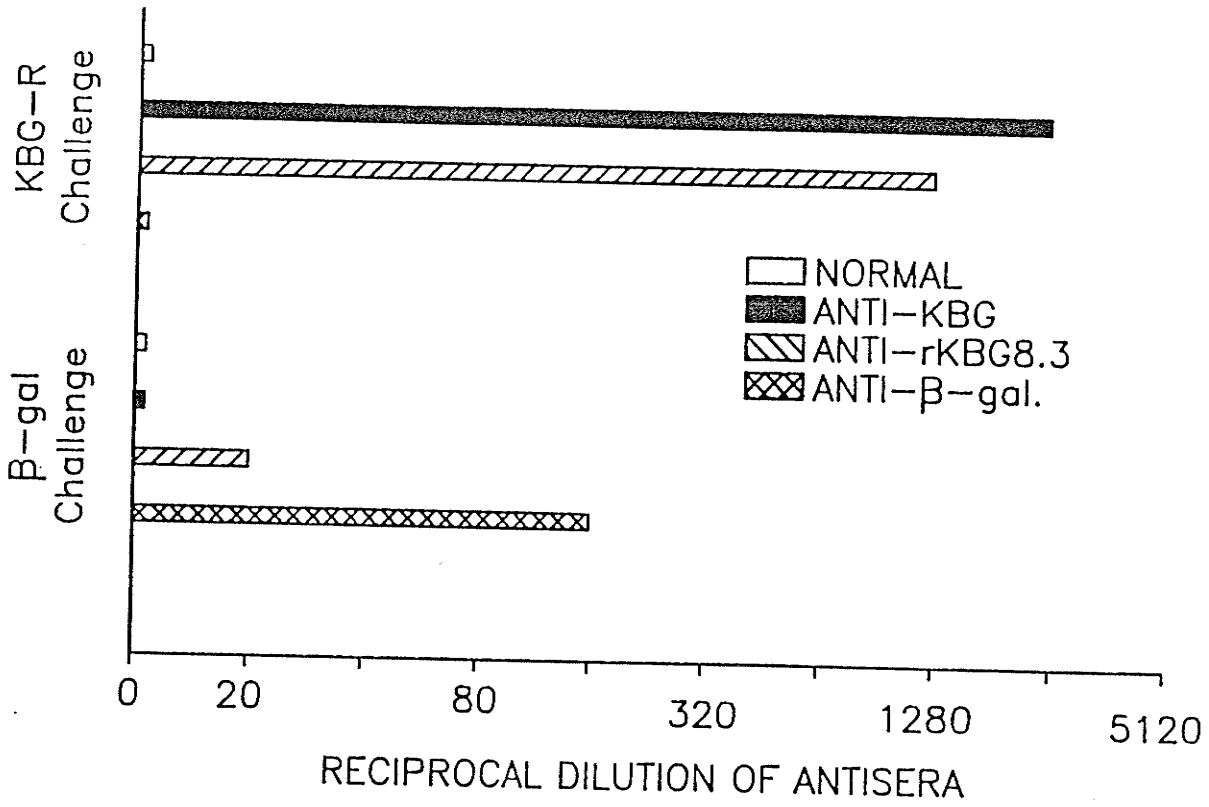

Fig. 1. Determination of specific IgE antibody titers by PCA assay. Two groups of rats were sensitized with the preimmune serum, antiserum to KBG, FP and β-galactosidase as marked and later challenged with either KBG proteins or β-galactosidase.

Table 2. PCA titers of sera preabsorbed with different antigens*

Antigens used for absorption	PCA titers	
	anti-KBG[a]	anti-rKBG8.3
KBG	40	0
rKBG8.3	640	0
β-galactosidase	2560	1280
non-treated control	2560	1280

* KBG pollen extract was used as challenge antigen.
[a] KBG stands for Kentucky Bluegrass pollen extract

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 β-galactosidase nor bacterial proteins were able to remove IgE antibodies specific to KBG allergens from antiserum to KBG proteins or the rKBG8.3 polypeptide. Similarly, the rKBG8.3 polypeptide was capable of absorbing IgE antibodies from antiserum to the rKBG8.3 or KBG proteins. The degree of absorption varied depending upon the serum. These results confirmed that the rKBG8.3 was capable of inducing KBG-specific IgE antibodies in mice.

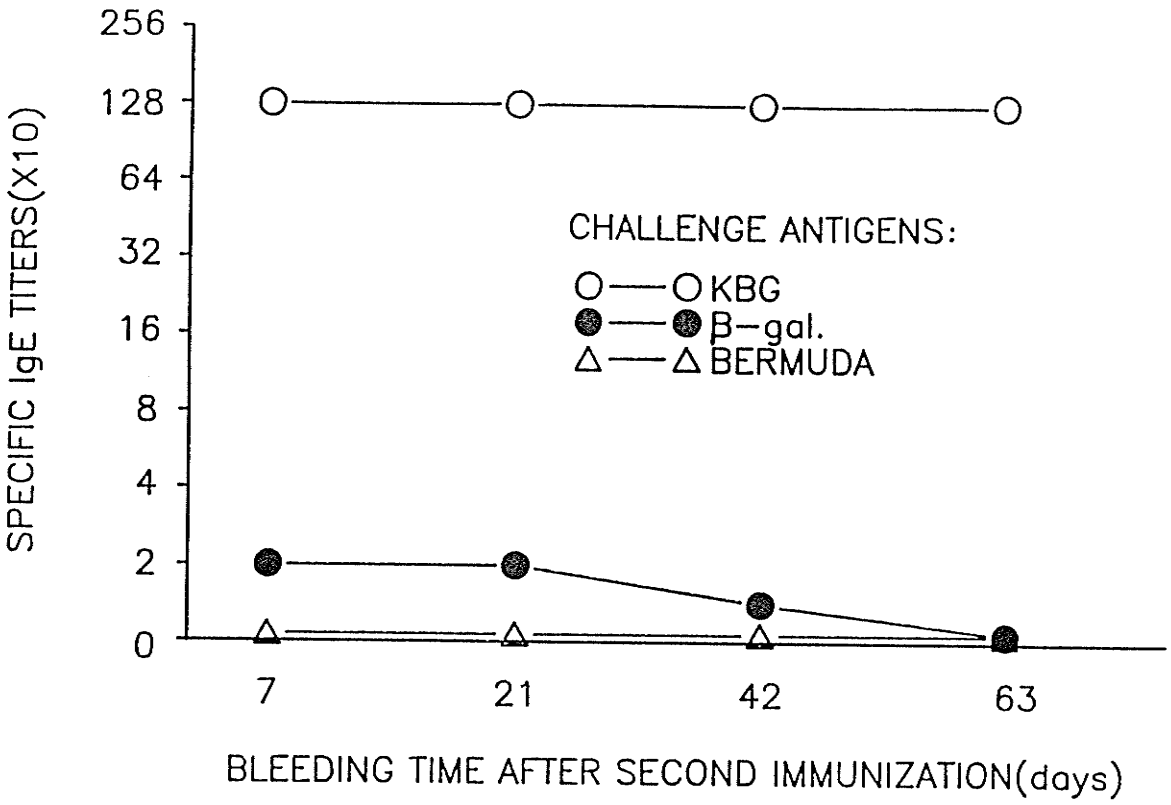

Fig. 2. Persistent IgE production to KBG proteins and β-galactosidase induced with rKBG8.3. IgE titers were determined using PCA assay at day 7, 21, 42 and 63 after second immunization.

Persistence of IgE antibodies induced with the rKBG8.3

Persistence of IgE antibodies induced with the FP was examined using the PCA assay. Sera from the same group of three mice were taken at three week interval and tested their IgE antibody titers (Fig. 2). The KBG-specific IgE antibodies persisted at high level (1:1280), and up to 63 days there was no apparent change in the titers. IgE antibodies to β-galactosidase, on the other hand, became undetectable at day 42. Bermuda grass pollen extract which possesses no cross-reactive epitopes with rKBG8.3 was used as negative control.

Inhibition of human IgE antibody binding 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 analyzed by employing an inhibition ELISA assay. Direct absorption with the rKBG8.3 polypeptide to remove the specific subset of IgE antibodies from the human sera reacting to KBG allergens and the full-length recombinant allergen, the rKBG60, demonstrated that around 20% and 80% IgE antibodies, respectively, were absorbed. Murine anti-rKBG8.3 sera inhibited human IgE antibody binding to the KBG and the rKBG60 allergens in a similar manner but to a larger extent than that of direct absorption with the rKBG8.3 polypeptide (Fig. 3). These results suggest that murine IgG antibodies recognize the

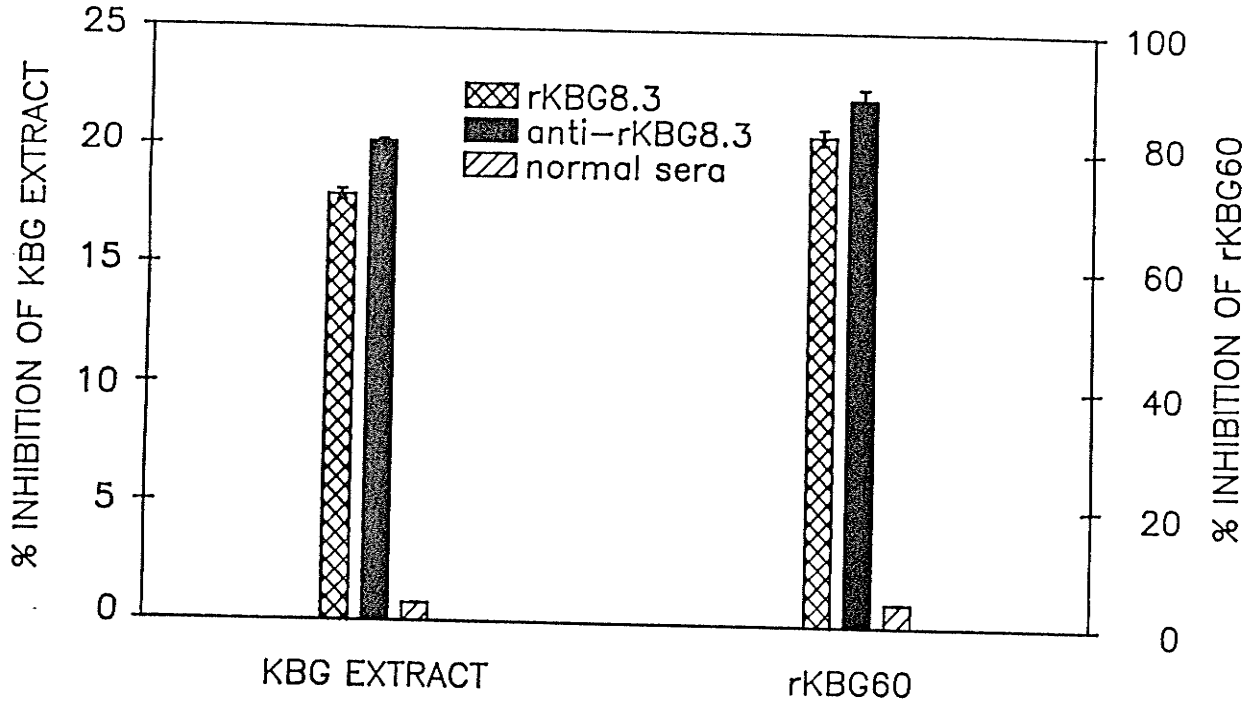

Fig. 3. Inhibition of human IgE antibody binding to KBG or Poa p IX (rKBG60) allergens with murine antiserum to rKBG8.3 as determined by ELISA.

same or adjacent epitopes to those recognized by human IgE antibodies.

In vivo cross-reactivities of the rKBG8.3 to allergens from other species of grass pollens

Cross-reactivity with allergens from seven different species of grass pollens with the rKBG8.3 was determined using PCA assay. After hooded rats were sensitized with antiserum to the rKBG8.3 or KBG pollen extract, they were challenged with different grass pollen extracts and PCA titers were determined (Fig. 4). Except pollen extract of Bermuda grass, all the other five grass pollen extracts possessed some components recognized by IgE antibodies against the epitopes on the rKBG8.3 and other allergens in KBG pollen. The antiserum to KBG pollen extract used as a positive control always gave higher PCA titers than that of antiserum to the rKBG8.3. Antiserum to β-galactosidase was used as a negative control. Furthermore, different grass pollen extracts exhibited varying PCA titers. These results demonstrated that IgE antibodies induced by one allergen, the rKBG8.3, were able to recognize allergens from five other species of grass pollens *in vivo*.

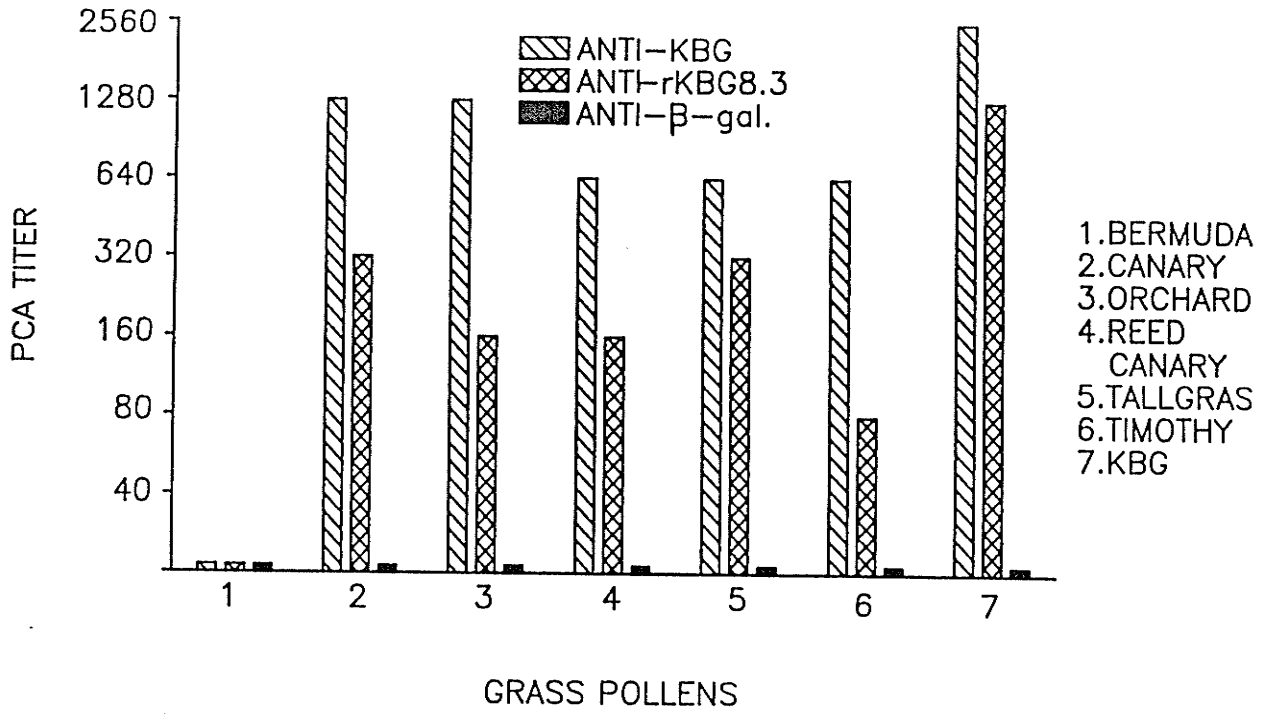

Fig. 4. Determination of in vivo cross-reactivity of rKBG8.3 with allergens in other species of grass pollens by PCA assay. Pollen extracts of KBG and Bermuda grass were employed as positive and negative controls, respectively.

DISCUSSION

The main objective of this study was to investigate the ability of recombinant allergen, the rKBG8.3, to stimulate KBG-specific IgE antibody production in mouse. These *in vivo* studies have provided evidence on (i) the immunogenicity of recombinant allergens, (ii) the magnitude and nature of the IgE immune response, and (iii) the cross-reactivity among grass pollens allergens in terms of *in vivo* IgE immune response. The KBG pollen allergens were used in this investigation representing the native Group IX allergens, since it was shown that anti-rKBG8.3 recognized at least three *Poa p* IX isoallergens (Chapter II and IV). However, in view of the possible cross-reactivity between Group IX and Group V allergens(7), it is likely that antiserum to the rKBG8.3 also reacts with group V specific proteins.

The FPs used for immunization comprised a 69 kDa truncated β-galactosidase fused to the rKBG8.3 (20.6 kDa) or the rKBG60.2 (8.9 kDa) allergenic peptide of *Poa p* IX allergens. Despite the difference in their molecular mass, the rKBG8.3 and the truncated β-galactosidase induced IgG antibodies with similar titers. Moreover, in terms of IgE response KBG-specific antibody titer was much higher than that of the β-galactosidase specific titers. By contrast, the rKBG60.2 representing an N-terminal fragment of the rKBG8.3, was unable to induce IgE antibodies, and it induced a low

titer of IgG antibodies reactive with KBG allergens indicating a weak epitope on this fragment. These results suggested that there exist T- and B-cell epitopes on both of these recombinant *Poa p* IX polypeptides, which is consistent with our previous observation(9). It is noteworthy that immunization of mice twice with the rKBG60.2 FP, followed by 2 injections with the rKBG8.3, led to a decrease in the titers from 1280 to 320 of IgE antibodies to KBG allergens (unpublished results). The non-allergenic fragments of allergens, therefore, have the potential of being exploited for allergen-specific immunotherapy.

Furthermore, β-galactosidase and the rKBG8.3 differ in their structure insofar that they induced differential IgE production. In the mouse model, IgE antibodies induced to KBG allergens after secondary immunization persisted at relatively high level and was not affected by repeated immunization. This is consistent with the scenario in atopic patients, whereby the production of IgE antibodies continues for a long period of 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 IL-4(15). This similarity between human and murine systems suggests that mice may serve as model for the manipulation of on-going IgE production. By contrast to the allergenic polypeptide, IgE antibodies specific to β-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. Using animal sera or antibodies as probes to study the B-cell epitopes of an allergen is much easier than employing limited amount and low antibody titer of human sera. Moreover, a previous study has indicated that epitopes of an *Amb a* III allergen were recognized by human, mouse and rabbit antibodies(16). Our results of inhibition assay are in agreement with the above findings presented in Chapter II and III, suggesting that mouse antibodies bound to the same or similar sites as the human IgE antibodies, although these data do 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 pollens also possessed components that are antigenically and allergenically cross-reactive with *Poa p* IX(8). 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 have significant implications in terms of future diagnosis and therapy of allergies using these recombinant allergens.

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CHAPTER VI

ANTIGEN- AND ISOTYPE-SPECIFIC IMMUNE RESPONSES TO A RECOMBINANT ANTIGEN-ALLERGEN CHIMERIC (RAAC) PROTEIN*

*: RAAC protein refers to the same fusion protein, rKBG8.3, the term used in the previous chapters of this thesis.

ABSTRACT

In order to investigate the possibility of modulating the antibody responses to an allergen by linking it to a non-allergenic molecule, we studied the fusion protein rKBG8.3 regarded as a recombinant antigen-allergen chimeric (RAAC) protein, which comprised of a truncated β-galactosidase and a *Poa p* IX polypeptide. Immunization of BDF1 mice with the RAAC protein in conjunction with dextran sulfate led to the differential antibody production. The *Poa p* IX fragment in the RAAC protein induced predominantly IgE antibodies, whereas the β-galactosidase fragment stimulated primarily IgG2a antibodies. The same pattern of antibody isotypes was observed when the individual components, the rKBG8.3 and the β-galactosidase fragment were used for immunization with dextran sulfate. Furthermore, the amount of the rKBG8.3 used for immunization, from 5μg to 100μg, showed no significant influence on the specific IgE antibody production. On the other hand, immunization of mice with these proteins in complete Freund's adjuvant induced no IgE antibodies either by the RAAC protein or by the β-galactosidase fragment; but low titer of IgE antibody was induced by the rKBG8.3. The RAAC protein and the β-galactosidase fragment induced IgG2a antibodies whereas the rKBG8.3 induced no detectable IgG2a antibodies. Furthermore, high titers of IgE antibodies were induced by the rKBG8.3 in dextran sulfate after the mice had been immunized twice with the protein in CFA; However, using the same strategy, insignificant *Poa p* IX-specific IgE antibodies were induced by the RAAC protein substituted for rKBG8.3.

INTRODUCTION

Allergens represent a class of antigens which, at extremely low doses, are capable of inducing the synthesis of IgE antibodies in genetically predisposed individuals. However, there is, so far, no direct evidence that allergens represent a structurally unique subset of antigens. The IgE immune response to allergens is believed to be primarily under the host's genetic control. Studies of MHC proteins of atopic versus nonatopic individuals and of experimental animals indicate that several loci are involved in the development of the IgE immune response (1-4). Some other genes are also believed to be involved in the immune responses to the allergens and determine the production of IgE antibodies(5-7). Thus, it is generally agreed that IgE immune responses are controlled by at least two sets of genes, one set of genes regulating the IgE responses and MHC class II genes regulating specific immune responses. The possible location of IgE regulating gene other than MHC may be on chromosome 11q region in human(6,7); however, this has been controversial and the precise location and sequence of any of this gene is as yet unknown.

It has been well demonstrated that T cells play a critical role in the induction of specific primary and secondary IgE immune responses(8-10). The functional analysis of two subsets of $CD4^+$ T helper cells, Th1 and Th2 cells, has revealed major differences in the mechanisms underlying IgE antibody production (11-14). In the murine model, whereas Th1 cells produce IFNγ, IL-2, and lymphotoxin

which favor IgG2a antibody responses, Th2 cells produce IL-4, IL-5, and IL-6, which promote the induction of IgE antibody responses. The effects of IL-4 and IFNγ are reciprocal, i.e., IL-4 induces the switch of B cells to IgE synthesis cells whereas IFNγ inhibits the effect of IL-4 and potentiates IgG2a synthesis(13,14). Using *in vitro* cultures of cloned T cells of humans, it was found that cells produce IL-4 when stimulated with the purified allergens, and secrete IFNγ when stimulated with non-allergenic antigens(15,16). While Th1 and Th2 cells represent mature phenotypes and are believed to originate from Th0 cells which secrete both IL-4 and IFNγ (17-20), the signal(s) triggering differentiation of the Th0 cells to these two types of functionally distinct mature T cells remains unknown.

To investigate the differential effects of antigens on the IgE and IgG antibody production, and to test the possibility of modulating an IgE antibody response to an allergen by linking it to a non-allergenic antigen, we examined the immunogenicity of a recombinant antigen-allergen chimeric (RAAC) protein, consisting of a truncated β-galactosidase(β-gal) and a cloned allergenic polypeptide, rKBG8.3, which represents a conserved region of the *Poa p* IX allergens of Kentucky bluegrass (KBG) pollen(21,22). The *Pao p* IX polypeptide was recently established in our laboratory to represent one of the major allergens of this grass pollen (21-24). Analysis of the antibody responses to the RAAC protein and its constituents revealed that the two constituents of the chimeric molecule induced different immunoglobulin isotype responses.

Moreover, the immune responses to these antigens was found to be greatly influenced by the adjuvant used.

MATERIALS AND METHODS

Synthesis of RAAC protein

The RAAC protein was synthesized in *E. coli* by ligation of the cDNA insert KBG8.3 into plasmid expression vector pWR590.1 (25) as described in detail in Chapter II.

Synthesis of the rKBG8.3

The KBG8.3 DNA insert in the above recombinant pWR590.1 plasmid was isolated by digestion with *EcoRI* and purified by agarose gel filtration as described elsewhere(26). The rKBG8.3 protein was produced and purified by employing pGEX vector system. Briefly, the insert was ligated in the modified pGEX 2T vector(27) and then transformed into JM105 cells. The fusion protein was expressed with IPTG induction and purified with glutathione agarose beads (Sigma, MO). Thrombin was used to cleave rKBG8.3 from the fusion protein when it was bound to the glutathione agarose beads and the rKBG8.3 was harvested by pelleting the beads.

SDS-PAGE and immunoblotting

Proteins isolated from the IgE binding clones were analyzed by a 10% SDS-PAGE gel and stained with 0.1% Coomassie blue. The separated proteins were also electrotransfered onto a nitrocellulose membrane. The membrane was blocked with 2% bovine

serum albumin in PBS buffer, and then probed with antisera (in dilutions of 1:2000) raised in mice against proteins of either KBG pollen extract or β-galactosidase. After three washes with PBS-Tween buffer, the antibodies bound to the polypeptides on the membrane were detected with alkaline phosphatase-conjugated goat anti-mouse IgG (Zymed, CA) and its substrate *p*-nitro blue tetrazolium chloride(NBT) and 5-bromo-4-chloro-3-indolyl phosphate(BCIP).

Immunization of mice.

Female, 6-8 week old, B6D2F1 mice (Jackson Laboratories, Bar Harbr, ME), were used for induction of immune responses to the proteins. The mice of different groups were immunized with RAAC protein, truncated β-galactosidase and different amounts of the rKBG8.3 protein, respectively, in dextran sulfate(DS)(Sigma, MO) or in complete Freund's adjuvant(CFA)(Sigma, MO). Each group consisted of three mice, and the experiments were repeated twice. Before immunization, the mice were bled and the sera were pooled and stored at -20^{0}C and the pool used a as negative control. Each mouse was primed with 5μg of the RAAC protein, or the β-galactosidase fragment. The rKBG8.3 was used for immunizaton at three different doses, 5μg, 50μg and 100μg per mouse. As adjuvant, 2.5mg dextran sulfate(DS) in 200μl saline or an equal volume of complete Freund's adjuvant(100μl) was used for the first immunization. After 23 days the mice were boosted with the same amount of the protein in 1mg dextran sulfate in 200μl of saline or in the equal volume of CFA.

The mice were bled and sera were collected after 7 days of second immunization, pooled within the group and stored at -20^{0}C. Antibodies were raised against KBG pollen extract and native β-galactosidase by immunization of the mice with the respective antigens in dextran sulphate as described before(26,28).

To examine modulation of the levels of allergen-specific IgE antibodies, the BDF1 mice were first immunized with RAAC protein or the rKBG8.3 in CFA, twice as described above for establishment of an immune response to the RAAC protein or to the rKBG8.3. The mice were then reimmunized with the same antigens in dextran sulphate for several times in an interval of three weeks. The sera were collected seven days after each immunization, and pooled within the group and stored at -20^{0}C. All the sera were analyzed at the same time for their antibody titers.

Cross-inhibition of the rKBG8.3 and the RAAC protein in binding to anti-KBG antibodies

The conservation of antibody binding abilities of the rKBG8.3 and the rKBG8.3 in the RAAC form was analyzed by the cross-inhibition between the two antigens in terms of their binding to murine anti-KBG pollen extract antibodies. Firstly, the titer of anti-sera to KBG pollen extract sera was determined. The rKBG8.3 and the RAAC protein were coated onto the ELISA microplates(Nunc, Denmark), with 0.1μg of the proteins in 100μl bicarbonate buffer, pH9.6, per well, at room temperature overnight. The remaining binding sites of the microplates were saturated with 2% BSA in

200μl PBS buffer by incubation at room temperature for 2 hours, after washing three times with PBS buffer containing 0.2% tween-20. Serially diluted antisera in a volume of 100μl against KBG pollen extract was added into the wells and incubated at room temperature for 2 hours. The plates were washed three times with PBS-tween buffer followed by the incubation at room temperature for 2 hours with 100μl of 1:2000 diluted goat anti-mouse Ig conjugated with alkaline phosphatase(Zymed, CA). The antibody binding was detected by enzymatic color reaction of p-nitrophenyl in 100μl alkaline phosphatase (AP) buffer at 37^{0}C for 30 minutes. The O.D. values were determined with ELISA reader at wave length 405. According to the dilution curve, 1:4000 of the antisera was used in the following inhibition assay. The inhibition assay was performed by ELISA essentially as described above, except that the antisera were used at a fixed dilution and incubation with different amount of antigens, ranged from 1μg to 400μg per ml, of either free the rKBG8.3 or the RAAC protein 1 hour preceded to the addition of the antisera into the wells as described before(26,28).

Detection of specific IgG1 and IgG2a and total antibodies

The KBG pollen allergen- and β-galactosidase-specific IgG1 and IgG2a antibodies were determined by using ELISA. The wells of microtiter plates (Nunc, Denmark) were coated separately with 1μg KBG pollen extract prepared as described previously (29) and 0.1μg β-galactosidase per well in 100μl carbonate/bicarbonate buffer, pH9.6, overnight at room temperature. After three washes with PBS-

tween buffer and saturation of the plates with 2% BSA in PBS buffer, 100μl of serially diluted sera were added to the wells, and the plates were incubated for two hours at room temperature, washed three times and then incubated with 1:2000 diluted alkaline phosphatase-conjugated goat anti-mouse IgG1 (Zymed, CA) or goat anti-mouse IgG2a (Zymed, CA). The wells were washed again three times and the enzyme substrate p-nitrophenyl was added in 100μl AP buffer and incubated for 30 min at 37^{0}C. The antibody titer was determined by the O.D. values which were three-fold higher than that of the pre-immune sera used as negative control in all experiments.

Detection of specific IgE antibodies

Antigen-specific IgE antibodies were determined by passive cutaneous anaphylaxis (PCA). The backs of three groups of Hooded rats, two rats in each group, were intradermally injected with 50μl of serially diluted preimmune and immunized murine sera. Forty-eight hours later the rats were challenged by intravenous injection of different antigens dissolved in 1.0ml of PBS containing 0.5% Evan's blue; one group was challenged with 1.0mg per rat of the KBG pollen extract, the second with 1.0mg per rat of Bermuda pollen extract, and the third with 0.5mg per rat of β-galactosidase (BRL, Missisauga, Ont). The amount of challenge antigens used here were determined with three different doses, i.e., for KBG pollen extract 0.5mg, 1.0mg, and 2.0mg per rat, and for β-galactosidase 0.2mg, 0.5mg, and 1.0mg per rat. The PCA titer of each serum was taken as

the reciprocal of highest dilution of the serum giving a clearly visible skin reaction (>5mm in diameter).

RESULTS

The RAAC protein

The RAAC protein was analyzed by SDS-PAGE electrophoresis and the western blotting(Fig. 1). As expected, the protein was detected in the lysate of the transformants with the recombinant plasmid and recognized by antiserum raised against either native β-galactosidase or the KBG pollen extract, whereas the lysate of the parent plasmid transformed cells containing the truncated β-galactosidase was only recognized by antisera to β-galactosidase. The rKBG8.3 was also synthesized in *E. coli* and recognized by the antisera to KBG pollen extract but not by the antisera to β-galactosidase sera(data not shown).

Antibodies induced with the RAAC protein in DS

BDF1 mice immunized with the RAAC protein in DS led to induction of both IgG and IgE type responses. To distinguish the two repertoires of specific antibodies induced with the RAAC protein, native KBG pollen extract and β-galactosidase were used as coating and challenging antigens in ELISA and PCA assays, respectively. Analysis of isotypes and subclasses of immunoglobulins induced with the RAAC protein revealed different spectrum of antibodies to β-galactosidase and KBG pollen allergens. As shown in Fig 2a, there was no significant difference in the

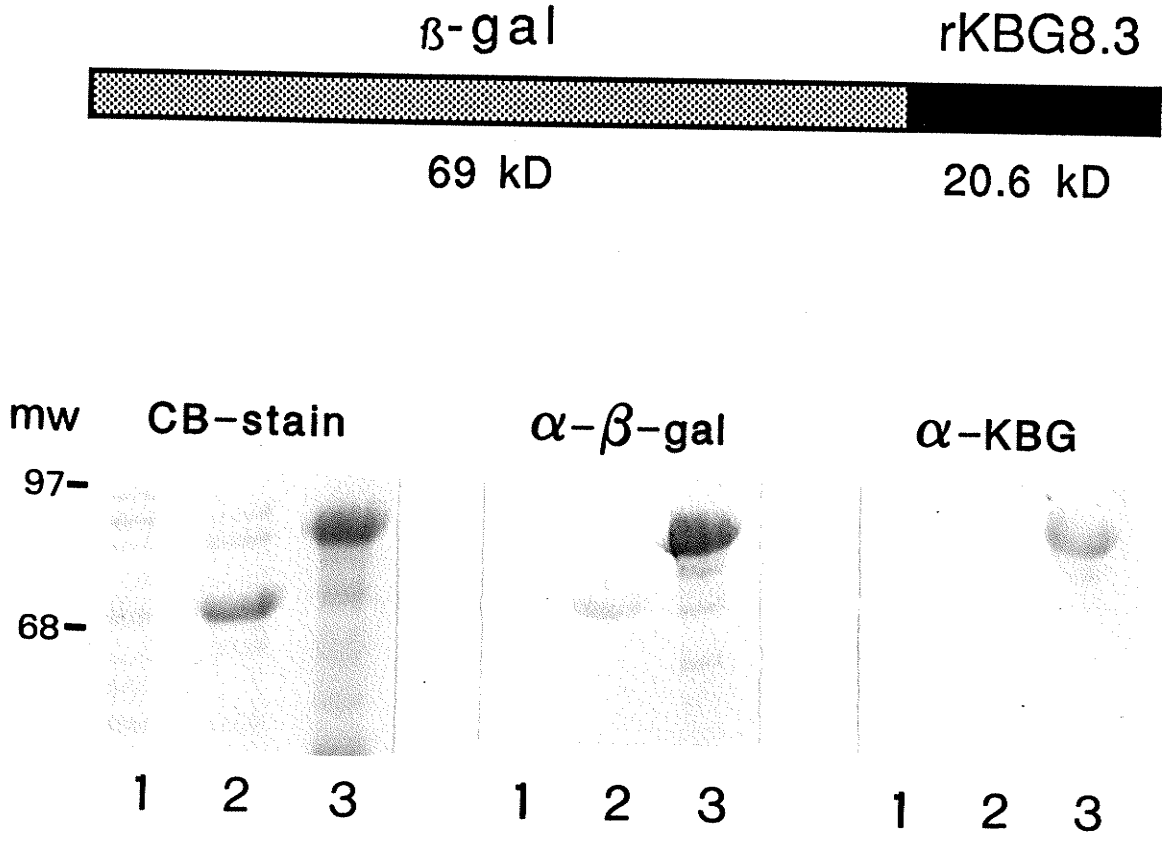

Fig.1 Schematic diagram of the recombinant antigen-allergen chimeric (RAAC) protein. (A) The protein consists of 69KD fragment of a common bacterial antigen, β-galactosidase, and a 20.6KD allergenic peptide of *Poa p* IX allergens from grass pollen. (B) SDS- PAGE electrophoretogram of the RAAC protein stained with Coomassie Blue (CB), and immunoblot of the gel developed with antiserum to β-galactosidase (α-β-gal.) or antiserum to KBG pollen proteins (α-KBG), as primary antibodies. Lane 1, 2, and 3 are the bacterial lysis from non-transformed, pWR590-transformed cells, and pWR590 containing rKBG8.3 insert transformed cells, respectively.

titers of the IgG1 isotype antibodies. However, the titers of IgG2a and IgE antibodies were strikingly different between the repertoires of the antibodies specific to β-galactosidase and those specific to KBG pollen extract. It was found that the IgG2a antibodies in the sera to β-galactosidase was about 1000 folds higher than that to KBG pollen allergens (Fig.2b). Conversely, the specific IgE antibodies induced with the RAAC protein were found mainly to KBG allergens (Fig.2c).

Conservation of antibody binding abilities between the RAAC protein and the rKBG8.3

The antiserum to KBG pollen extract recognized both RAAC protein and the rKBG8.3, and titers of the antibodies in response to these proteins are similar, i.e., up to 1:64000. The inhibition of binding to the RAAC protein or the rKBG8.3 to the anti-KBG sera by the rKBG8.3 or the RAAC protein was observed even at a concentration of 1μg/ml, but for complete inhibition, a total of 400μg protein in 1ml of 1:4000 diluted sera was required. The results of cross inhibition with the concentration of inhibitor at 400μg/ml are shown in Fig.3. While each protein inhibited itself completely to bind to the antiserum to KBG pollen extract, the RAAC protein and the rKBG8.3 abolish also the binding of the antiserum to each other, respectively. The bacterial protein at 400μg/ml had no noticeable effect on the antisera to bind to the RAAC protein and the rKBG8.3. These results indicate that the protein encoded by the same cDNA clone KBG8.3, when synthesized in the fusion form, retained its antibody binding ability.

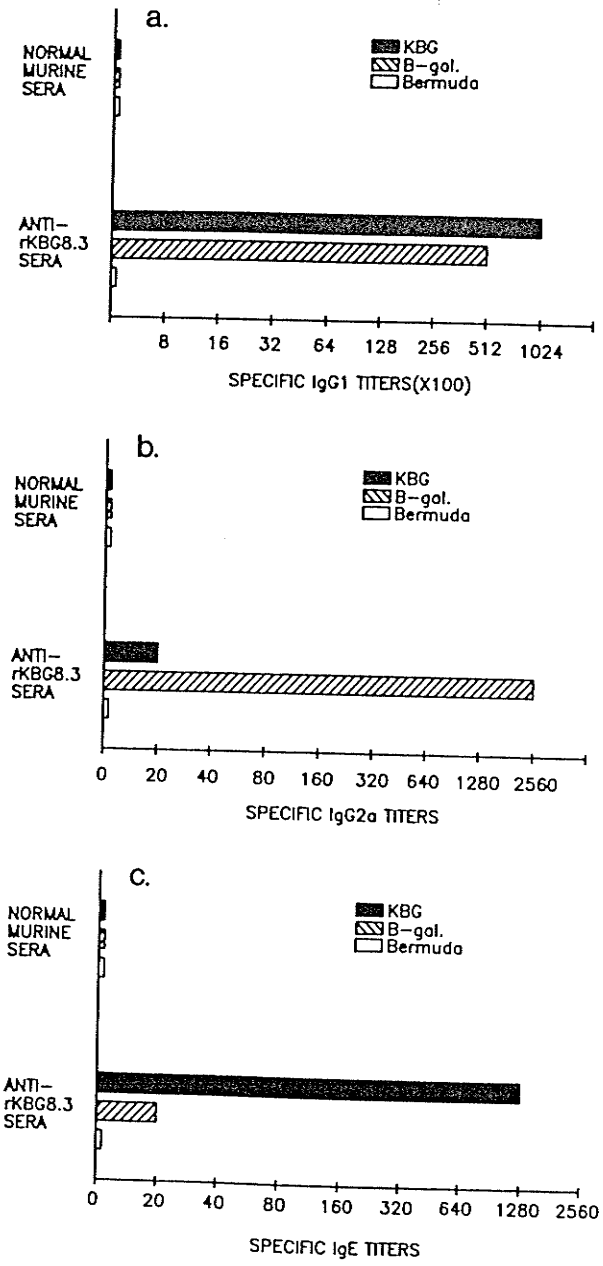

Fig.2 Detection of IgG1, IgG2a and IgE immunoglobulins specific to β-galactosidase and *Poa p* IX pollen allergens induced by RAAC protein. The IgG1 (a) and IgG2a (b) immunoglobulins were detected by ELISA employing alkaline phosphatase conjugated anti-murine subclass Ig antibodies. The titers of IgE (c) antibodies were determined by PCA assay.

Antibodies induced with the rKBG8.3 and the truncated β-galactosidase in DS

The antibodies induced with rKBG8.3 and the truncated β-galactosidase are shown in Table 1. It can be clearly seen that the rKBG8.3 and the truncated β-galactosidase were highly immunogenic and induced high titers of IgG1 antibodies. The difference was observed between IgE antibodies and IgG2a antibodies induced by the two antigens. The rKBG8.3 at amount of 5.0μg per mouse induced IgE antibodies up to the titer of 1280, whereas truncated β-galactosidase induced IgE titer only up to 80. In contrast to the IgE antibodies induced by the two antigens, truncated β-galactosidase induced

TABLE 1. SPECIFIC ANTIBODIES INDUCED AFTER IMMUNIZATION WITH rKBG8.3 ALLERGEN AND β-GAL* IN DEXTRAN SULPHATE

Ags used in immunization	Antibody titres		
	IgG1	IgG2a	IgE[1]
rKBG8.3[2]	655360	NIL	1280
rKBG8.3(50ug)	2621440	320	1280
rKBG8.3(100ug)	1310720	160	1280
Truncated β-gal[3]	2621440	10240	80
Normal sera to rKBG8.3	NIL	NIL	NIL

*β-galactosidase.
[1] The IgE titers were determined with PCA assay, the other antibody titers were determined with ELISA.
[2] The coating antigen is KBG pollen extract, and each mouse in this group was immunized with 5ug antigen. The other groups immunized with different amount of antigen were indicated.
[3] The coating antigen is native β-gal, and each mouse in this group was immunized with 5ug antigen.

high titers of IgG2a antibodies, whereas the rKBG8.3 induced no detectable IgG2a antibody even at 1:20 dilution of the antisera. Similarly, the rKBG8.3 applied at doses of 50ug and 100ug per mouse induced high titers of IgG1 and IgE antibodies but low titers of IgG2a antibodies. These results led to the inference that the two constituents of the RAAC protein induced the same pattern of antibody immune responses as that of the RAAC protein, that is both antigens induced high titers of IgG1 antibodies, but the ability to induce IgE and IgG2a antibodies were significantly different.

Antibodies induced with the RAAC protein and the rKBG8.3 and the truncated β-gal in Complete Freund's Adjuvant(CFA)

The pattern of antibody isotype production by the RAAC protein and its constituents was also examined in conjunction with complete Freund's adjuvant. When the RAAC protein was used in CFA, high titers of IgG1 and IgG2a antibodies to both KBG pollen allergens and β-galactosidase were observed. However, IgE antibodies were not found in the sera to either components of the RAAC protein. Similar pattern of antibody isotypes was also found in the sera from the mice immunized with the truncated β-galactosidase in CFA, i.e. the sera contained high titers of IgG1 and IgG2a antibodies but no detectable IgE antibodies. In contrast to the above findings, the rKBG8.3 in CFA induced no detectable IgG2a antibodies, although similar high titers of IgG1 antibodies were induced. Moreover, the rKBG8.3 in CFA induced detectable IgE antibodies as determined by

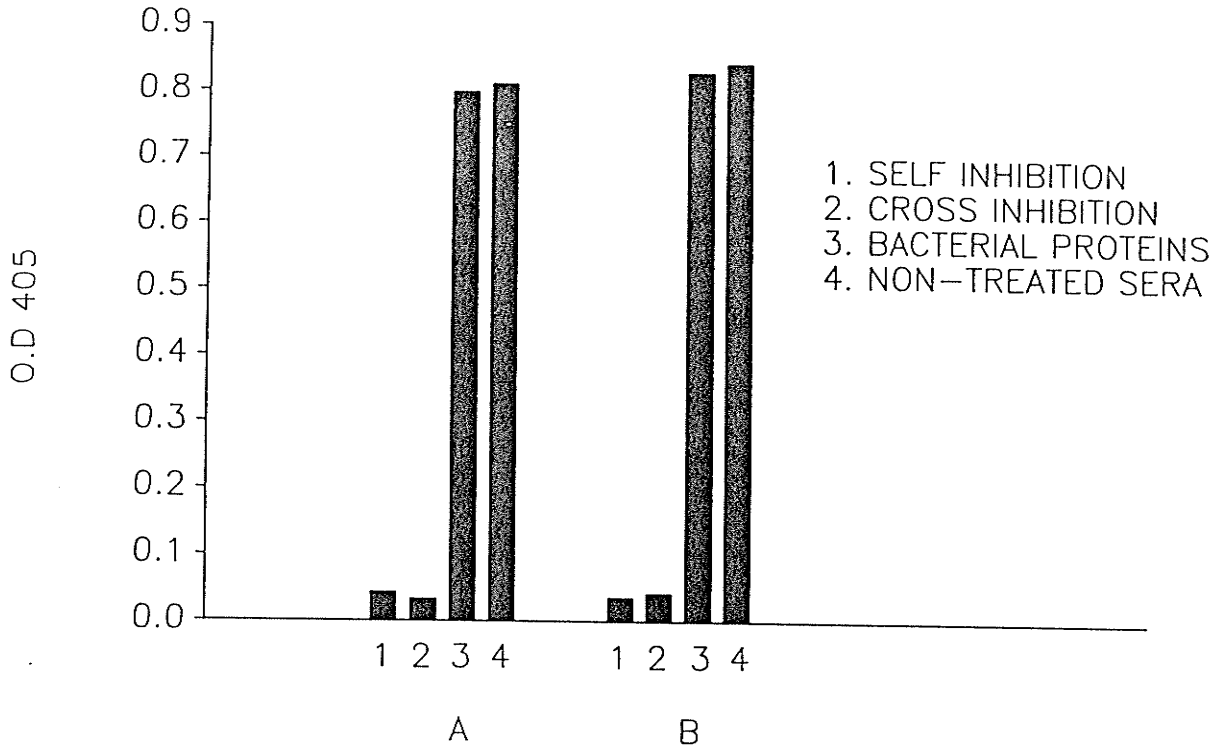

Fig.3 Cross-inhibition of the RAAC protein and the rKBG8.3 to bind to anti-KBG pollen protein extract. The RAAC protein and the rKBG8.3 were coated four triplicate wells. The sera were incubated with the different antigens before addition into the wells. Among the antigens, the self and bacterial proteins were used as positive and negative controls.

PCA assay. These results, summarized in Table 2, indicated that CFA as an adjuvant enhanced IgG2a antibody production but inhibited IgE antibody production specific to the RAAC protein which contains also β-galactosidase. However, the CFA did not exert similar effects on the rKBG8.3 alone. These results suggest that a given adjuvant may influence on induction of antibody isotypes differentially dependent upon the antigens used.

TABLE 2. SPECIFIC ANTIBODIES INDUCED AFTER IMMUNIZATION WITH RAAC PROTEIN AND ITS INDIVIDUAL COMPONENTS IN CFA

Ags used in immunization	Antibody titres		
	IgG1	IgG2a	IgE[1]
RAAC PROTEIN[2]			
Poa p IX specific	20480	20480	NIL
β-gal* specific	40960	40960	NIL
rKBG8.3	327680	NIL	40
β-gal.	327680	163840	NIL
Normal sera to β-gal	20	NIL	NIL

*** β-galactosidase.**
[1] The IgE titers were determined with PCA assay, the other antibody titers were determined with ELISA.
[2] The two repertoires of the antibody specificities induced with the RAAC protein were determined with different coating antigens.

Modulation of IgE antibody production by immunization of mice with the RAAC protein in CFA

Immunization of mice with the RAAC protein in CFA, led to the induction of allergen-specific IgG2a antibodies, whereas immunization with the rKBG8.3 induced undetectable IgG2a

antibodies. In order to investigate the possibility of induction of IgE antibodies in these mice, the mice were further immunized with either the RAAC protein or the rKBG8.3 in dextran sulfate. The results are shown in Fig.4.

The group of mice, after immunization with RAAC protein in CFA, showed no detectable IgE antibodies to KBG pollen allergens. The mice were then challenged with the same protein but in dextran sulfate, the IgE antibodies were found with a 1:20 dilution of the serum. Further injection of the protein and dextran sulfate increased slightly the IgE titer to 1:80, and then it was stable even after third immunization. The IgG1 and IgG2a titers specific to both KBG allergens and to β-galactosidase, and the IgE antibodies specific to β-galactosidase were also determined after each immunization by ELISA and PCA assays, respectively. The results showed there was no significant change in IgG1 and IgG2a titers to both of the antigens. Furthermore there was no detectable IgE antibodies to β-galactosidase (data not shown). The mice immunized with the rKBG8.3 in CFA twice showed high IgE antibody production after further injections with the allergenic polypeptide in dextran sulfate. The IgE antibody titer increased from 40 to 640 after first immunization of the mice with the rKBG8.3 in dextran sulfate, and kept at this level after second immunization.

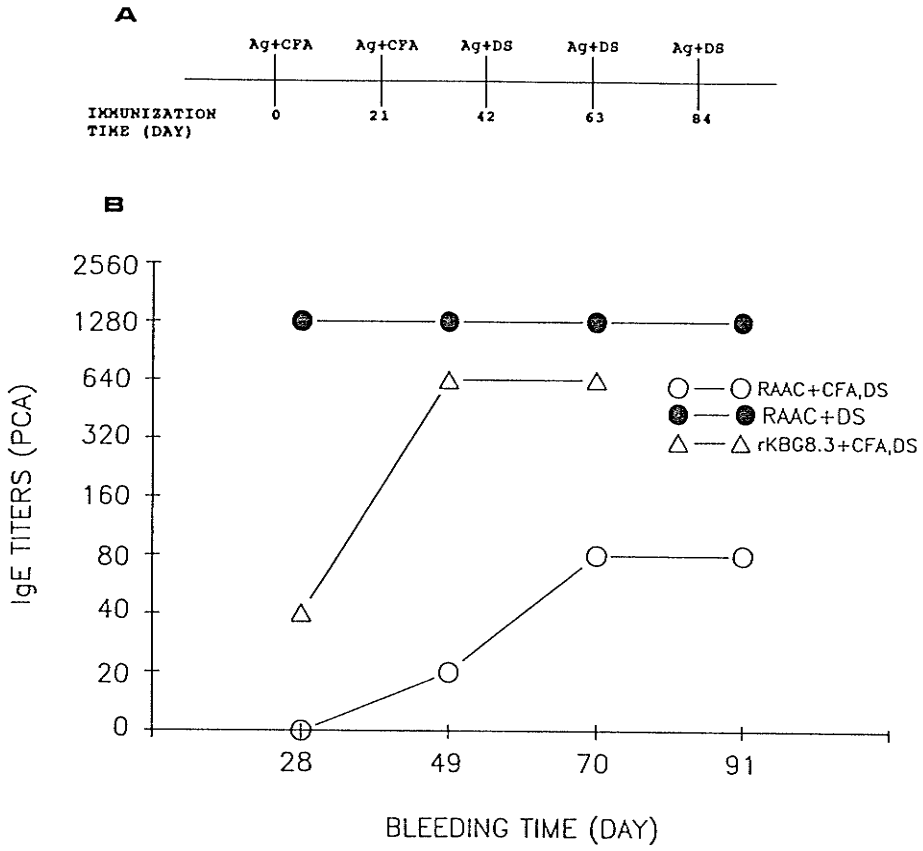

Fig.4 Modulation of IgE antibody production. The mice were first immunized twice with either the RAAC protein or the rKBG8.3 in complete Freund's adjuvant, and then challenged with the same protein but in dextran sulfate at three week intervals, as shown immunization schedule 4A. 4B shows the changes of the IgE antibody titers determined by PCA assay, and also the IgE titers of the mice immunized with the RAAC protein and DS alone was used as control.

DISCUSSION

The RAAC protein comprising the N-terminal half of β-gal(residue 1-590) and C-terminal fragment of *Poa p* IX allergens essentially represents two different antigens in terms of induction of immune responses in both human and animals. The β-gal, a common bacterial antigen, has been used to investigate the T-B cell interactions(30-32). In BDF1 mice, it was found that whereas C-terminal fragments of β-gal provided T cell help, the N-terminal fragments favoured suppression(31). To our best knowledge, there is no report so far that this antigen induces IgE antibodies and causes immediate hypersensitivity naturally in human and experimentally in animal models. On the other hand, the *Poa p* IX allergens have been shown to represent a major group of grass pollen allergens and 90% of patients allergic to grass pollens possess IgE antibodies specific to this allergen in their sera(24,33). Thus, the RAAC protein provides useful model for study of immune responses to different regions of a molecule, and the possibility of modulation of IgE responses to the allergen with the non-allergenic antigen.

We have previously reported that there is no cross-reactivity between KBG pollen antigens and β-galactosidase (26,28). However, immunization of mice with the RAAC protein induced high titers of antibodies to both β-gal and KBG pollen allergens; This observation demonstrated that both components of the RAAC protein possess epitopes capable of stimulating immune responses. Whether these

epitopes in the two components of the RAAC protein have different capacities in terms of stimulation of different antibody responses, resembling the natural immunization in human, was examined by the analysis of the specific isotypic antibodies to the different antigens. The remarkable difference in their ability to induce either specific IgE or IgG2a antibodies to the two constituents of the RAAC protein implies that one component induced predominantly a Th2 type immune response while the other produced mainly a Th1 type response. The lack of significant difference between the titers of specific IgG1 antibodies induced by the two components supports the findings that, unlike IgE antibodies, the synthesis of IgG1 antibodies are not tightly controlled *in vivo* by the IL-4 and IFNγ lymphokines(34).

The same pattern of immune responses induced by the RAAC protein was also observed when the two individual components were employed. The substantial differences between the constituents in their abilities to induce the antibody isotypes under the same experimental conditions suggest that the differences in structure of these two antigens determine the directions of the immune responses in the animals with the same genetic background. These results support the recent findings that in the same individual some T cell clones are Th1 type with specificities predominantly to the bacterial antigens, and others Th2 type with specificities mainly to the allergenic proteins(15,16). Furthermore, the patterns of immune responses induced by the two components of the RAAC protein and the individual components immunized separately were

essentially the same, these results strengthen the notion that distinct epitopes on an antigen may regulate the immune response(31).

Interestingly, the rKBG8.3 immunized with DS showed no dose dependent induction of specific IgE antibodies, which is not in agreement with the previous observations that the low doses of the antigens preferentially induced IgE antibodies whereas high doses mainly induced IgG type antibodies(35,36). However, high doses of the rKBG8.3 induced also low titer IgG2a antibodies, while β-gal was also found to induce low titer IgE antibodies when immunized with DS. Thus, under certain conditions antibodies of all isotypes may be produced upon a given antigen stimulation. In our case, the large amount of the rKBG8.3 and co-injection of the truncated β-galactosidase with DS appeared to contribute to the low titers of specific IgG2a and IgE antibody production, respectively.

It has been reported that there exists hierarchy among linked epitopes with respect to induction of immune responses(37). The RAAC protein employed in conjunction with DS in this report, however, showed no such hierarchy between the two components. The discrepancy may be due to the fact (i) that the protein we used has multiple epitopes involved and therefore is more complicated; (ii) that the adjuvant DS may play a role in enhancing IgE immune responses and inhibiting the IgG2a responses. Thus, whereas the truncated β-gal could not fully express its ability to elicit the Th1 type response, the IgE induction properties of the *Poa p* IX polypeptide was magnified.

The role of the adjuvant in induction of immune responses was also examined by employing the CFA as adjuvant. Unlike the RAAC protein in DS alone, the RAAC protein in CFA used for immunization induced high titers of IgG2a but virtually no IgE antibodies to both components. These results suggest that the isotypes of antibodies to a given antigen are determined by the adjuvant used in immunization. However, the results from the two individual components, the rKBG8.3 and the truncated β-gal, suggest that the adjuvant can only influence but not determine the antibody isotype immune responses to different antigens which may in fact determine the direction of the responses. This conclusion is consistent with the recent reports that some polypeptides induce IgE antibodies independent of the adjuvant used(38,39). The reason that high titers of IgG2a antibodies induced also to the allergenic components by the RAAC protein was presumably due to the fact that β-gal possesses the structure favoring the Th1 type response and CFA enhanced this properties. Furthermore, the smaller *Poa p* IX allergenic polypeptide linked to the β-gal, but not in free form, induced also a Th1-type response. The precise mechanism underlying this phenomenon is as yet unclear. It is likely that the IFN-γ released by the β-gal-specific T cells may influence the isotypes of antibodies induced by the allergen.

The possibility of specific down-regulation of IgE antibody production was tested in mice immunized with the RAAC protein or the rKBG8.3 with CFA prior to injection of the same antigen in DS. Although both the RAAC protein and the rKBG8.3 were able to

increase the IgE antibody production when administered with DS after sensitization in the presence of CFA, the substantial difference in IgE titers between them indicates (i) β-galactosidase influences the IgG2a antibody production to the linked *Pao p* IX polypeptides, (ii) IgE antibody production is not affected significantly by the pre-existence of IgG1 antibodies, but is significantly affected by the pre-existence of IgG2a antibodies.

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CHAPTER VII

GENERAL DISCUSSION

AND

CONCLUSIONS

Application of techniques of molecular biology to studies of protein allergens has had a major impact on advances in our knowledge of the primary structures of allergens(1-9). The availability of amino acid sequences of allergens has facilitated further studies of their T- and B-cell epitopes(4,10). Moreover, gene expression procedures have made it possible to obtain virtually unlimited quantities of purified allergens and defined allergenic fragments from the cloned genes. The possible biological functions of some of these cloned allergens were also defined by sequence analysis(1,3,8).

In the present study, the synthesis of the recombinant allergen, rKBG60, and of its overlapping fragments have facilitated (i) the analysis of epitopes of this grass pollen allergen, (ii) the investigations of its cross-reactivities with other grass pollen allergens, (iii) the establishment of a murine model for induction of IgE antibodies to recombinant allergens, and (iv) the regulation of allergic immune response *in vivo*.

Despite advances relating to the cellular and molecular basis of IgE synthesis and in the analysis of structure-activity relationships of several allergens, the mechanisms underlying IgE antibody production in atopic patients remain to be elucidated. Production of IgE antibodies to protein allergens is mainly determined by two equally important factors: (i) genetic predisposition, i.e., the host's genetic background, and (ii) environmental factors, i.e., exposure to culprit allergens and

including possibly also some adjuvant-like materials. It is to be stressed that genetic studies to-date indicate that a number of genes may be involved in the development of allergic immune response. These studies suggest that gene-environmental interactions are of pivotal importance in allergies. Of the environmental factors, allergens have been always suspected to play a critical role in determination of IgE antibody production in atopic patients(11-13). However, as Baldo and Donovan pointed out(13), we are in the paucity of the substantial data concerning (i) the chemical compositions of allergenic determinants, (ii) the number of allergenic sites on an allergen, (iii) antigenic relationships between allergenic determinants on proteins from the same and from the different sources, and (iv) relationship between IgE and IgG binding determinants. Therefore, whether allergens possess some features to selectively elicit and bind IgE antibodies remains as yet unknown.

Allergenic epitopes have been reported, like epitopes of other antigens, to be either conformational or sequential in nature(14-16). In our studies, the mapping of antibody binding epitopes of the allergen was accomplished by using comprehensive strategies of overlapping peptides which comprised (i) relatively long peptides synthesized with the aid of the corresponding cDNAs, (ii) overlapping 20 residue peptides synthesized by automated methods, and (iii) overlapping short 10 residue peptides synthesized by the pin method. With these overlapping peptides we have defined both linear and conformational epitopes of the rKBG60 allergen. Greene

et al(10) successfully employed the overlapping peptide strategy to analyze IgE binding epitopes of *Der p* I allergen and concluded that at least 30 amino acid residues were required for the epitopes of this allergen to bind IgE antibodies. This was consistent with previous finding that *Der p* I possessed mainly continuous, topographically conformational epitopes. Our results also revealed one continuous, topographically conformational epitope of the rKBG60 allergen as determined by using two sets of overlapping synthetic peptides. However, the majority of the epitopes of the rKBG60 allergen appear to be sequential in nature. It should be pointed out that in the absence of the knowledge of the crystallographic analysis of antigen-antibody complexes, definition of the epitopes of the rKBG60 may be considered tentative. However, since the epitopes analyzed by immunochemical methods have been essentially the same as those found by X-ray crystallographic analysis(18), the information provided by this study regarding the epitopes of this grass pollen allergen is of profound importance in terms of devising epitope-based therapies.

The IgE binding epitopes of the rKBG60 appear not to be restricted to IgE antibodies. Most of them also react with murine and human IgG antibodies, although some discordance was observed. Thus, our results are in general agreement with the findings of studies of other allergens that epitopes of allergens do not selectively recognize IgE antibodies only(10,17-19). However, whether the amino acid residues of the epitopes involved in the interaction with IgG and IgE antibodies are the same is uncertain

at this stage. Availability of human IgE and IgG monoclonal antibodies to the defined allergenic epitopes may help resolve this issue.

To determine whether antigens may have some intrinsic properties, which make them allergenic, we further utilized the RAAC protein for induction of antibodies in mice. These results indicate that antigens and allergens may be different in their ability to induce IgE antibody production in a given individual or in animals with the same genetic background. The results of this study clearly demonstrate that the RAAC protein, injected in conjunction with a appropriate adjuvant, induced the alteration of the immune responses, IgG2a response, to the allergen. The precise cellular and molecular mechanisms underlying this observation remains to be elucidated. However, on the basis of the existing knowledge of the T cell control of immune response, these results may be explained as follows. One possibility is that the T cell epitopes of the *Poa p* IX polypeptide stimulated the T cells in presence of CFA to differentiate into Th1 cells. Another possibility is that the IFN-γ secreted by the Th1 cells specific to the β-galactosidase resulted in the B cells specific to the *Poa p* IX polypeptide to switch to the IgG2a secreting cells. The first explanation, although supported by recent findings that Th1 and Th2 epitopes have no gross differences (20), appears not to be the case because the rKBG8.3 does not induce substantial IgG2a in presence of CFA. The lymphokines secreted in the microenvironment, therefore, may explain this non-cognate help (21).

Notwithstanding the unknown mechanism underlying this alteration, our results clearly revealed that induction of the allergen-specific IgG2a antibodies prevents the production of IgE antibodies. These observations are consistent with the recent finding that polymerized allergens induce IFN-γ that inhibits specific IgE responses(22).

The results that antigens or their epitopes may be distinguished on the basis of their interaction with Th1 or Th2 cells, as was the case for β-galactosidase and rKBG8.3 in this study, suggest that the structures of epitopes are critical to the development of Th cell repertoire. This is in agreement with recent reports that in the same atopic patient there exist two compartments of T cells, Th1 and Th2, which showed different specificities respectively to common bacterial antigens and allergens(23-25). It is worth noting in this context that the same antigen may possess both Th1- and Th2-type epitopes. Recently, using *Poa p* IX allergen-specific human T cell clones it was demonstrated that the T cell clones which recognized the peptides containing T-cell epitopes exhibited in general a Th2 phenotype. However, the clones recognizing 20-residue peptide #26 or clones induced by peptide #26 were of Th1/Th0 phenotype (S. S. Mohapatra & S. Romagnani, unpublished data).

The therapeutic potential of recombinant allergens and/or their peptides is currently under intense investigations by many academic and industrial laboratories. An elegant approach to abolish the allergic reaction by using monovalent IgE binding

epitopes was developed based on the mechanism that histamine release of the mast cells or basophils requires the bridging of the IgE antibodies on the cell membrane(26,27). The results from this study and others(10,17-19), which indicate that a number of epitopes of an allergen are capable of binding IgE antibodies, suggest that it may be difficult to use a few monovalent allergenic epitopes to prevent the bridging between the IgE antibodies. Another approach for the treatment of allergies utilizing T cell epitopes of allergens may be similarly ineffective since pollen allergens have been shown to possess many T cell epitopes(19,31); thus the *Poa p* IX allergens were found to comprise at least seven T cell epitopes in this study. Furthermore, the studies of the *Poa p* IX and other allergens(19) revealed that the majority of the B- and T-cell epitopes were present on the same short peptides; therefore, one would not expect that the risk to elicit allergic reactions with these peptides could be eliminated.

On the other hand, it may be feasible to achieve specific and favourable immunotherapeutic effects by using the defined allergenic fragments, which possess the major epitopes. Our results demonstrated that the rKBG60 allergen has an uneven distribution of human IgE-binding epitopes. Studies of another *Poa p* IX-like allergen of ryegrass pollen supports this finding, although it was concluded that the IgE binding epitopes of that allergen are solely on its C-terminal region(28). Also, the cloned *Lol p* I allergen appears to have its major T cell epitopes on the C-terminal region as determined by T cell proliferation(4). Therefore, it may be

possible to make immunotherapeutic reagents with the small fragments, such as rKBG8.3, instead of large intact proteins.

Furthermore, the reports that grass pollens contain a number of allergenic proteins shed doubt on the strategy of specific immunotherapy using individual allergenic constituents. However, the allergens, such as from grass pollens, can be classified into several groups no matter which species they are from(29). The intra- and inter-species cross-reactivities among the allergens(30) from the different grass pollens indicate that it may be possible to treat the patients who are found allergic to most grass pollens (except some pollens, such as Bermuda and Johnson grasses) with a few defined major allergens. Moreover, the well-defined allergenic fragments containing the major epitopes of the main allergens could be linked together and then fused to a non-allergenic protein. This chimeric antigen could then be used for the purpose of immunotherapy of predisposed individuals.

In summary, we have characterized immunochemically a recombinant allergen corresponding to a major group of grass pollen allergens, the group IX allergens. The results suggest that a significant improvement may be achieved in relation to specific diagnosis and therapy of allergies with recombinant allergens and/or their peptides(31). The following summary statements are considered to represent **original contributions to immuno-allergology** made by the author during this study:

1. Three allergenic proteins were identified in the crude extract

of KBG pollen with the aid of antibodies raised against the rKBG8.3, with molecular masses of 28KDa, 32KDa and 34KDa, which correspond to the three full-length cDNA clones, KBG41, KBG60 and KBG31, respectively.

2. The recombinant allergen rKBG60 and its four overlapping polypeptides were synthesized by *E. coli* using an overlapping cDNA expression strategy.

3. Antibody-binding analysis of the recombinant polypeptides revealed that although the B cell epitopes were distributed over the whole molecule, the majority of the IgE and IgG binding epitopes were localized in the C-terminal regions, represented by rKBG8.3, which is conserved in all cloned *Poa p* IX isoallergens.

4. Mapping of antibody-binding epitopes with two sets of synthetic peptides revealed that the rKBG60 possessed at least (i) 13 epitopes reactive with murine IgG antibodies, (ii) six epitopes recognized by human IgG antibodies, and (iii) four human IgE-binding epitopes which were mainly located in the C-terminal region of the molecule.

5. Immunization of mice with the synthetic peptides demonstrated that most but not all antibody-binding epitopes were coincident with T cell epitopes in the rKBG60 allergen.

6. On examination with the aid of murine anti-rKBG8.3 and human IgE antibodies, components cross-reactive with *Poa p* IX allergens were found in 10 of 12 other species of grass pollens.

7. Differences between rKBG8.3 and truncated β-galactosidase, even when fused together on one molecule, in their capacity to induce, respectively, specific IgE and IgG2a antibodies were demonstrated in mice.

8. The RAAC protein comprising two major clusters of epitopes, represented by the β-gal and the *Poa p* IX allergenic polypeptide, induced different types of immune responses to the constituent peptides depending upon the adjuvant used.

9. Down-regulation of IgE antibody responses to the *Poa p* IX allergen was achieved by immunization of mice with RAAC protein in the presence of CFA.

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