THE UNIVERSITY OF MANITOBA

# IMMUNOLOGIC CHARACTERIZATION OF A RECOMBINANT KENTUCKY BLUEGRASS ALLERGEN

BY

MING YANG, M.D.

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## ABSTRACT

A recombinant allergen (RA) of Kentucky bluegrass pollen was synthesized as a fusion protein ( FP ) in *Escherichia coli* by cDNA cloning procedures and was compared with its natural counterparts with respect to its allergenic properties. The 81 kDa band of FP in *E. coli* extract was purified by excision of the band from SDS-PAGE and solubilized. On western immunoblot, only the 81 kDa band which corresponded to FP showed IgE binding reactivity. This RA was demonstrated to bind to the IgE antibodies of 90% of 55 grass pollen allergic individual sera. A positive correlation was observed between the IgE antibody levels of RA and KBG or grass-pollen extracts. However, sera from five non-atopic individuals did not demonstrate any detectable IgE binding reactivity to KBG and RA. The observations that KBG significantly inhibited the binding of human IgE Abs to RA and *vice versa* were interpreted to indicate that the RA possessed several human IgE binding sites. In another study, 978 allergic patients' sera examined by ELISA for RA specific IgE antibodies, 39.4 % of these shared IgE binding sites. Also close relationship was shown between KBG and RA in IgE and IgG4.

RA was also evaluated for its immunogenicity in terms of its capacity to stimulate proliferation of mice PLN cells *and human T cells in vitro.* Comparable

degrees of lymphocyte proliferation were consistently obtained on stimulation with either KBG or RA of PLN cells from mice immunized with KBG or RA. This study demonstrated that the RA retained the immunogenicity of the original KBG pollen in terms of its ability to ( i ) induce the *in vivo* priming of PLN cells and ( ii ) elicit the *in vitro* proliferation of the antigen-primed PLN cells.

In order to determine epitopes on RA recognized by human T cells, peptides were synthesised according to the RA sequences. Proliferation assay utilizing PBMCs from allergic patients demonstrated that T cell epitopes were located at least two regions on RA, which were recognized by T cells. Allergen-specific human T cell clones were also established *in vitro*. But T cell response to Ag requires the presence of an antigen-presenting accessory cell. This study demonstrates that B cells in the form of EB virus-transformed B lymphoblasts are able to present allergen to human T cells and induce proliferation of these cells.

Collectively, our results suggest that the RA encoded by cDNA KBG7.2 which we have synthesized constitutes a major allergenic constituent of grass pollens and may be of diagnostic and therapeutic value.

LIST OF ABBREVIATIONS:

| | |
|---|---|
| Ab: | Antibody |
| Ag: | Antigen |
| APC: | Antigen presenting cell |
| BSA: | Bovine serum albumin |
| CD: | Cluster of differentiation |
| cDNA: | Complementary DNA |
| Con A: | Concanavalin A |
| cpm: | Counts per minute |
| CSIF: | Cytokine stimulation inhibitory factor |
| EBV: | Epstein Barr virus |
| ELISA: | Enzyme-linked immunosorbent assay |
| FACS: | Fluorescence-activated cell sorter |
| FCA: | Freund's complete adjuvant |
| FCS: | Fetal calf serum |
| FP: | Fusion protein |
| GM-CSF: | Macrophage colony stimulating factor |
| IFN: | Interferon |
| IL: | Interleukin |

| | |
|---|---|
| IT: | Immunotherapy |
| LMW-BCGF: | Low molecular weight B cell growth factor |
| KBG: | Kentucky blue grass |
| MHC: | Major histocompatibility complex |
| mRNA: | Messenger RNA |
| M.W.: | Molecular weight |
| O.D.: | Optical density |
| PBS: | Phosphate buffered saline |
| PBMC: | Peripheral blood mononuclear cell |
| pI: | Isoelectric point |
| PLN: | Popliteal lymph node |
| *Poa p*: | *Poa pratensis* |
| RA: | Recombinant allergen |
| SDS-PAGE: | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SPT: | Skin prick test |
| TCR: | T cell receptor |
| TBS: | Tris buffered saline |

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# I. INTRODUCTION

## 1. IMMUNOPATHOLOGY AND THERAPY OF IgE-MEDIATED ALLERGIC REACTIONS

The induction of allergen-specific IgE antibodies and the activation of polymorphonuclear granulocytes characterize the allergic immune response in genetically predisposed atopic individuals (sensitive individuals who would respond with allergic symptoms on natural environmental exposure), which comprise 25-30% of the total population (1). Primary exposure to allergens leads to the production of allergen-specific IgE antibodies which then become attached via their Fc receptors to the corresponding receptors on the patient's mast cells and basophils. When sensitized airways or the skin of atopic subjects is provoked by exposure to the specific allergen, a biphasic response often occurs (2).

The classic immediate hypersensitivity allergic reaction is initiated by the binding of multivalent allergen to the specific-IgE antibodies bound to the high affinity Fcε receptors of mast cell and basophil (3). Bridging of IgE receptors on these cells leads to perturbation of the cell membrane and within minutes results in a complex sequence of biochemical events occurring both at the surface and within the mast cell and basophil granulocyte, leading to cellular activation,

arachidonic acid metabolism, and release of mediators, such as histamine. These mediators rapidly induce the characteristic clinical features of the allergic reaction, including itching, sneezing, rhinorrhea, stomach cramps, bronchospasm, wheezing and urticaria (4).

The late phase of the allergic response occurs four to eight hours after the initial mast cell-dependent reaction (4). Mast cell plays no direct role in the late-phase reaction but is indirectly responsible for migration of other cell types (5). During this response the reaction site are infiltrated by eosinophils, neutrophils, macrophages, lymphocytes and basophils which subsequently release secondary mediators including prostaglandins, leukotrienes B4 and C4, and related activating factors that are formed only upon degranulation of mast cells (6, 7). With the subsequent cellular infiltration into the original site of an allergic event, a smouldering late-phase reaction is induced. Thus, present evidence indicates that IgE antibodies are responsible not only for the immediate allergic reaction but also for the subsequent development of the late phase of an allergic reaction (8, 9). The production of IgE antibodies specific to allergenic molecule (s) is one of the most critical events in the development of allergic diseases.

Immunotherapy (IT) has constituted a major form of treatment for allergies since 1911, when it was first introduced by Freeman (10). IT involves administration of increasing doses of a specific extract comprising allergens to which the sensitive (atopic) individual would respond with allergic symptoms on

natural environmental exposure (11), leading to the induction of allergen-specific IgG. It is assumed that these IgG antibodies, also referred to as "blocking antibodies", prevent binding of the allergen molecules to cell-bound allergen-specific IgE antibodies, leading to the reduction of allergic symptoms. It has been shown that passive transfer of allergen-specific IgG can indeed provide effective protection against exposure to allergen (12). This assumed " blocking antibody " activity of IgG seems to be a reasonable explanation for the observed protection.

Crude pollen extracts, used in IT, are heterogeneous mixtures of allergenic and non-allergenic components. Thus, IT will be affected if there are irrelevant allergens in the extract, because within crude pollen extracts the total concentration of relevant ones is decreased. Omitting an allergen that plays a major role in the disease process also will minimize the response. Purified and well characterized allergens, therefore, are essential for either using them directly for IT or for developing other rational approaches for IT, such as modified allergens, referred to as allergoids. Furthermore, analysis of the immunologic features of the allergens such as the identification of their B and T cell epitopes are important, since they (i) may lead to the development of new forms of diagnosis and therapy and (ii) may lead to understanding the mechanism of IgE synthesis.

# 2. IMMUNOLOGIC CHARACTERIZATION OF ALLERGENS

## 2. 1. Characterization of allergens

Allergens are usually high molecular weight proteins or glycoproteins present in pollen, house dust, food, animal dander, hair, urine, mites, insect venoms and other environmental products, which are capable of eliciting the formation of specific IgE antibodies.

The immunogenicity of a given allergen or antigen depends upon the following three factors: (i) the processing of the molecule to its immunogenic fragments by antigen-presenting cells, (ii) the exposure of these fragments in association with major histocompatibility molecules on the surface of these cells, and (iii) their presentation to the appropriate T-cells capable of recognizing the resulting complex (13). On the basis of the above, it is reasonable to assume that the allergenic molecules comprise determinants (epitopes) which are capable of recognizing: (i) the IgE and IgG antibodies on the B cells, (ii) the MHC molecules on the antigen presenting cells, and (iii) the T cell receptors on the T cells. However, the mechanism as to how the allergenic determinants are involved, either directly or indirectly, in the regulation of the specific IgE synthesis remains to be elucidated. Such studies have been limited due to the lack of primary sequence information of allergens and the unavailability of pure allergenic proteins in sufficient quantities.

Pollens constitute a major group of aeroallergens because of multiplicity of constituents contributed by tree, weed and grass pollens and the fact that exposure to these allergens is unavoidable. All investigated grass pollen extracts which are complex mixtures of proteins can be resolved into 30 or more different antigenic components, some of which have the capacity to induce synthesis of IgE antibodies and cause atopic allergy in susceptible individuals. Aqueous grass pollen extracts contain many antigens of which several have been shown to be of major importance in grass pollen allergy. Within each grass extract, allergen can be divided into at least five groups, depending on their physicochemical and immunochemical characteristics. It is possible to detect immunochemically similar components in pollen extracts from different grasses.

So far, allergenic content of protein extracts from pollens of varies plant species have been difficult to standardize. During protein extraction, important allergenic components may be lost for a number of reasons, like instability of proteins, protease activities in the extracts, an variation in protein content from batch to batch. For example, mite extracts from commercial sources were found to lack a major mite allergen. A number of attempts have been made to produce purified allergenic molecules from pollen and other sources by standard protein biochemistry techniques (14) as gel chromatography, isoelectric focusing, affinity chromatography, and allergen specific monoclonal antibodies (15). Although these methods have proven somewhat useful, they are time consuming and labour

intensive and are not adequate to produce pure allergens in large quantities. Impurities of preparations of allergens made it very difficult to establish precise amino acid sequences or molecular structures of the epitopes of the allergens which are recognized by T helper and B cells.

2. 2. Application of recombinant DNA (rDNA) technology to characterization of allergens

To overcome these difficulties, several laboratories have recently employed rDNA procedures for the molecular characterization of major allergens. Thus, the mRNA from the corresponding aeroallergens were isolated, reverse transcribed to produce complementary cDNAs. These cDNAs were cloned in expression vector λgt11; The cDNAs encoding allergens were identified, sequenced to deduce the primary structures of allergens. The cDNAs were also cloned in high-level expression vectors for the production of recombinant allergens (RA). The rDNA technology has proven to be a potent tool to characterize allergens. After the cDNA sequence of allergens is obtained, mapping of B cell and T cell epitopes is substantially facilitated, and peptides can be synthesized according to the deduced amino acid sequences.

A list of diverse aeroallergens, the primary structure by which have been deduced by the rDNA procedures is summarized in Table 1. RAs can be purified in unlimited quantity and therefore can be used (i) in studies to understand what

Table.1 A current list of recombinant allergens

| Source | IUIS* Name | SDS-PAGE M.W.(kDa) | High-level expression** | Reference |
|---|---|---|---|---|
| Mites | | | | |
| | Der p I | 26.0 | Yes | 16 |
| | Der p II | 15.0 | Yes | 17 |
| | Der p | 14.0 | Yes | 18 |
| Vespid | | | | |
| | Dol m v | 23.0 | Yes | 19 |
| Pollens: | | | | |
| Ragweed | | | | |
| | Amb a I | 37.0 | Yes | 20 |
| | Amp a II | | Yes | 21 |
| | Amp t V | 4.4 | Yes | 22 |
| Grasses | | | | |
| | Lol p I | 24.0 | Yes | 23,24 |
| | Poa p IX | 33.0 | Yes | 25,26, 68 |
| Trees | | | | |
| | Bet v I | 17.4 | Yes | 27 |
| | Profilin | 12.0 | Yes | 28 |
| Cat | | | | |
| | Fel d I | 38.0 | No | 29 |

* International union immunological societies.

** Protein expression more than 51% of total cell protein was considered as high-level expression.

contributes to the dysregulation of the immune system leading to enhanced IgE-synthesis which is characteristic for atopic patients; (ii) in immunotherapy; (iii) in diagnosing allergic diseases, including skin testing, the serum specific IgE measurement and (iv) in standardization of allergens. Individuals allergic to one grass pollen may also suffer from allergic reactions following exposure to other grasses not indigenous to the local where the sensitization occurred, as a result of cross-reactivity among allergenic molecules. The extensive cross-reactivity among allergens may be used to an advantage, since it may be expected that the preparation of suitable tolerogenic derivatives of the allergens of one grass pollen could result in effective suppression of the IgE antibody responses to the cross-reactive allergens present in other grasses. The availability of synthetic allergens will also facilitate the investigation of T cell responses to several allergens which may have several implications for the treatment of allergies. Similarly, the means to engineer modified allergens for therapy from a knowledge-base of epitopes is approaching (31).

Information on the nature of epitopes of antigens recognized by B and T cells is complex. B cells can recognize natural antigen which does not need processing. A majority of B cell epitopes are thought to be conformational (32). Recognition by T cells requires processing of antigen and presentation of its short linear peptides complexed with the MHC molecule. The consensus of opinion is that peptides of less than 12 amino acids can bind to T cell receptors when

presented in the context of the appropriate MHC molecule. To understand the mechanisms underlying production of IgE antibodies it is essential to determine allergenic and antigenic sites, as well as the sites responsible for recognition of allergens by T cells.

Most of the RA expressed in *E. coli* may not possess tertiary structure, solubility, and immunologic properties of the original protein. It is therefore, important to purify RA and characterize the RA in terms of its ability to bind with IgE antibodies, and evaluate its capacity to stimulate T cells of sensitized donors. Such studies are limited because of the relatively recent advent of RAs. Of particular relevance to this thesis is the immunologic characterization of a recombinant allergenic molecule from KBG pollen.

## 3. REGULATION OF IgE ANTIBODIES

### 3. 1, The role of T cells in IgE production

The roles of allergen-specific IgE and mast cells are well established in the etiology of immediate type hypersensitivity. Although it has been apparent for many years that T-cell help is required for the production of IgE by B cells (33), only recently has it been possible to begin a careful dissection of the precise role played by T cells. Such analysis was made possible by the advent of T-cell cloning and the availability of recombinant cytokines and blocking Abs specific for these

cytokines (2). In the course of an immune response to an antigen, B cells are activated as a consequence of two main signals provided by T cells: the first is mediated by physical interaction between T and B cells, and the second by T-cell-derived (or exogenous) cytokines which then act upon B cells inducing them to differentiate (34). Both the T cell contact and the lymphokine signals are produced by T cells following MHC class II restricted antigen-specific cognate interaction between T and B cells (35). Both functional and biochemical studies have demonstrated that the generation of cellular immune responses depends upon antigen receptors on T cells (TCR) recognizing peptidic fragments of foreign proteins associated with products of MHC, that are expressed on the membrane of accessory cells (36).

In 1986, Mosmann and co-workers (37) proposed a major subdivision of mouse CD4 positive helper T (Th)-cell clones based on differences in their pattern of cytokine production. T-cell clones of the Th1 subset synthesize and secrete interleukin 2 (IL-2) and gamma-interferon (IFN-$\gamma$), but not IL-4 or IL-5, whereas T-cell clones of the Th2 subset produce IL-4 and IL-5, but not IL-2 and IFN-$\gamma$. Today it is clear that there exists a third population of T cells, named Th0, which may represent precursors of the Th1 or Th2 CD4 positive subpopulations (38). Data provided by S. Romagnani (39) confirmed the evidences that T cells with stable Th1 or Th2 patterns existed in humans. With a large series of human T-cell clones exhibiting clear Th1 or Th2 phenotypes, he demonstrated that human Th1

and Th2 clones differ in their cytolytic potential and mode of help for B-cell Ig synthesis.

Del Prete and collaborators found that both recombinant IL-4 and IL-4-containing supernatants are ineffective in inducing IgE synthesis in highly purified tonsillar B-cell suspensions containing less than 1 % T lymphocytes and monocytes (40). The IL-4 dependent IgE synthesis can be restored by the readdition of appropriate concentrations of autologous or allogenic T cells, and the inclusion of monocytes potentiates this response (41, 42). Direct evidence that a physical interaction between T and B cells is required for IL-4-dependent IgE synthesis to occur is provided by assaying IgE synthesis in a double chamber system. When T and B cells are cultured in different chambers separated by a millepore membrane that is permeable to molecules but not to cells, IL-4 can not induce the synthesis of IgE. IgE synthesis occurs only when T and B cells are cultured in the same chamber (43, 44). Kinetic studies have provided evidence that the physical interaction between B cell with T cell is required before the signal provided by IL-4. IgE synthesis does not occur when B cells are cultured with IL-4 for three to four days before the addition of T cells, while enhancement of IgE synthesis is obtained when the cells are co-cultured before the addition of IL-4 (44, 45).

3. 2. The role of cytokines in IgE production

Early studies in rodents suggested that IgE production could be regulated by antigen-specific helper and suppressor T cells, and by isotype-specific factors showing affinity for IgE (the so-called IgE-binding factors) (46). Recently a further pathway of IgE regulation, essentially based on the cytokines, which influence IgE production, early mediator release, and late phase inflammatory responses, have been studied in detail (2).

The reciprocal activity of IL-4 and IFN-$\gamma$ on synthesis of IgE Abs, has been discovered in mice (47,48). Further research has provided substantial information on the mechanisms of regulation of human IgE synthesis. It is well established that IL-4 and IFN-$\gamma$ are the main regulatory cytokines of IgE production, with opposite effects on the synthesis of this immunoglobulin (49). Studies in both mouse and human systems have shown that the mechanism for IL-4 induce IgE production involving signalling the B cell to switch from the production of IgM to the production of IgG1 and IgE in the mouse, or to IgG4 and IgE in human (50). However, other roles of IL-4 are the induction of class II MHC antigen, the low affinity receptor for IgE (CD23), and an increase of the synthesis of IgE (51). More recently, IgE production was shown to be more effectively inhibited by anti-IL-4 receptor antibodies than by anti-IL-4 antibodies (52).

The role of other T cell-derived lymphokines in the production of IgE have

also been studied; IL-6 may provide a signal to enhance ongoing IgE synthesis, since it upregulates Ig production in an isotype-nonspecific fashion (53). Interestingly, D. Vercelli *et al.* found that endogenous IL-6 is essential in IL-4-dependent IgE induction, since anti-IL-6 antibody completely inhibited the production of IgE induced by IL-4 (54). In similar experiments, another T-cell product, IL-5, was shown to increase IL-4-dependent IgE production (55). Other studies found that the addition of anti-IL-2 antibody to the culture consistently exerted a strong inhibitory effect on the IL-4 induced IgE synthesis. Suggesting that IL-2 plays a role in the IL-4 -mediated IgE synthesis (56). The presence of a T cell-derived low molecular weight B cell-growth factor (LMW-BCGF) is also necessary (57). LMW-BCGF, recently cloned (58), supports the growth of certain populations of B cells and is also involved in Ig production (2).

Cytokines can also increase the release of mediators from human basophils. (51). Moreover, IL-3 is able by itself to induce a direct release of histamine from blood basophils, but others find that IL-3 and other cytokines such as IL-5, IL-6, GM-CSF, and BCGF only act as a priming agent. since they do not themselves possess a direct histamine releasing activity but increase the histamine releasability after anti-IgE stimulation of the cells (2).

The effects of T cell derived lymphokines are not limited to the up-regulation on IgE production; some lymphokines are capable of negatively regulating IgE synthesis. Interferons (IFN gamma and IFN alpha) appear to be involved in the

regulation of IgE *in vivo*. Both of them block the IgE synthesis, possibly by interacting with IL-4 induced responses (51). A recently described T cell-derived cytokine, called cytokine stimulation inhibitory factor (CSIF, now termed IL-10), has been described as having similarly negative effect on IFN-$\gamma$ production by mouse T cells (59).

These data combined indicate that the cytokine net work is essential for the regulation of the IgE synthesis as summarized in Figure 1 (60).

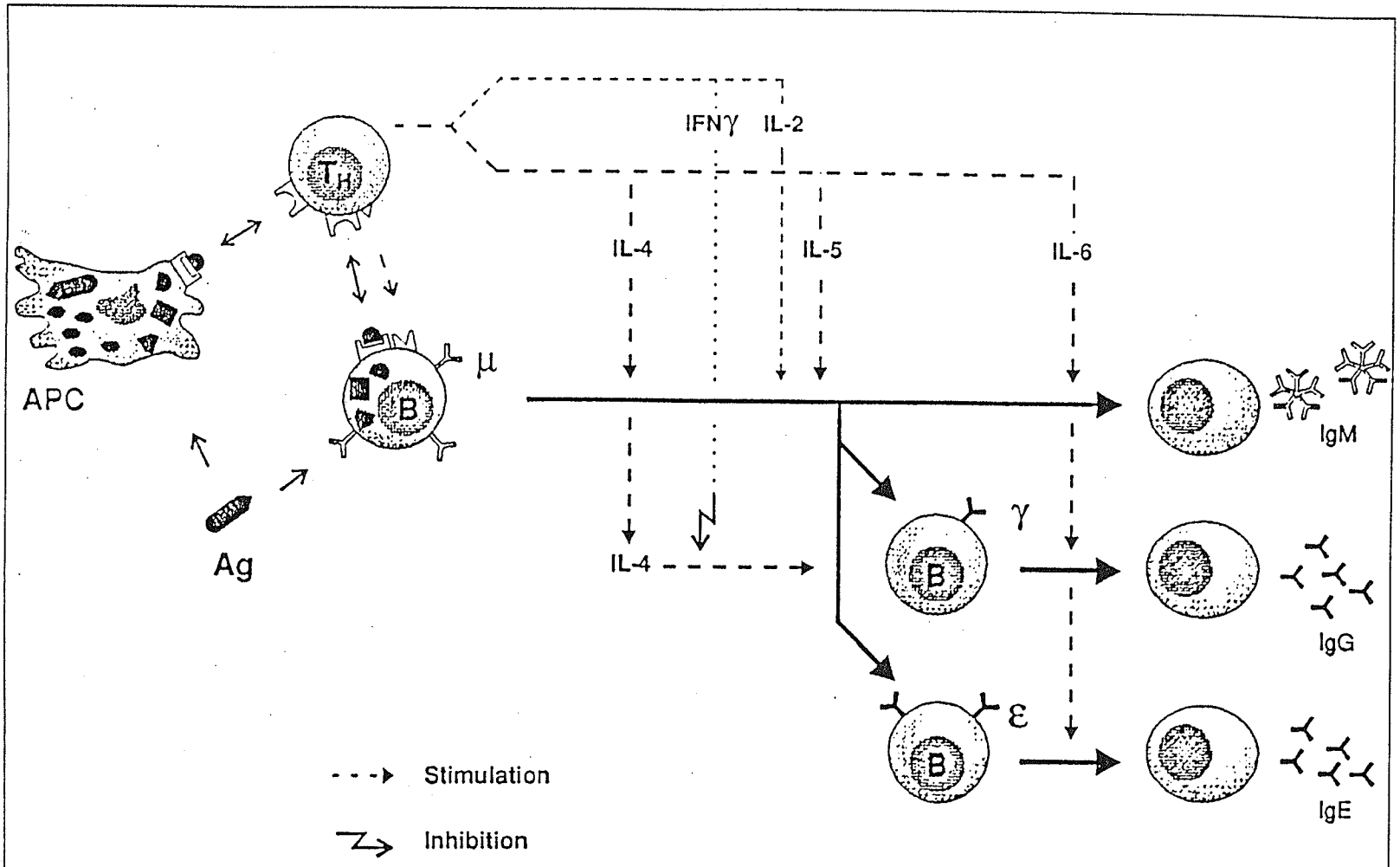



Figure 1.
Schematic representation of mechanisms which regulate an immune response leading to specific IgE antibody formation (Ag antigen; APC antigen presenting cell).

Fig. 1

## II. BACKGROUND AND OBJECTIVES

We have been investigating the allergens of Kentucky bluegrass (KBG) (*Poa pratensis* L.) pollen, which is one of the main pollens leading to respiratory allergies in the Canadian Prairies, other parts of North America, and Europe. Previously KBG pollen extract was reported to contain a multiplicity of antigenic and allergenic components with a wide range of molecular sizes and charges (61-63). Moreover, this complex mixture of components was reported to possess either haptenic or allergenic and antigenic properties (64-66). The observations that the P.I. value of allergenically active components ranged from 3 to 10 provide the example of the heterogeneity of the components present in KBG pollen (67).

To investigate further the allergenic and antigenic properties of the KBG allergens, our laboratory resorted to rDNA procedures. Briefly, mRNA was isolated from the KBG pollen and then using this mRNA a cDNA library was constructed in the phage expression vector λgt 11. Screening of this library with a pool of six sera from allergic patients, led to the isolation of cDNA clones. Several positive clones were detected; three homologous full length cDNA clones, KBG 31, 41 and 60, encoding a major group of KBG pollen allergens were reported (26). These clones exhibit an exceptionally high degree of similarity among them but had no homology with other known proteins or genes. (Fig. 2)

(26).

One of these cDNAs was designated as KBG7.2 which corresponded to the C-terminal of the full length cDNA clones and was further analyzed. The peptide encoded by KBG7.2 was expressed using the plasmid vector pWR590.1 in *E. coli* as a fusion protein in association with $\beta$-galactosidase (68). On the basis of the above and other immunological studies, it was concluded that the above cDNA clones encode a group of proteins which represent a new and previously uncharacterized group of grass pollen allergens, which have been designed as *Poa p* IX (26, 69). For this studies, the RA encoded by the cDNA KBG7.2 was employed.

**Hypothesis:**

RA was synthesized in *E. coli* by recombinant DNA procedures which was identified by its binding with human IgE antibodies. Because the cDNAs were synthesized from mRNA of KBG pollen, it was hypothesized that (i) the RA could be used to identify the corresponding KBG pollen proteins, and (ii) this RA would be equivalent to its counterparts in KBG pollen, in terms of its allergenic, antigenic and T cell recognition properties. The humoral and cellular immunologic methods were employed to prove our hypothesis.

**The main objectives of the present study are:**

( i ) to characterize and compare the recombinant allergen, KBG7.2 with its natural counterpart (s) with respect to its ability to bind human IgE and IgG4 antibodies;

( ii ) to test RA's ability to stimulate proliferation of murine PLN cells from immunized mice.

( iii ) to characterize human T cell responses to grass allergens including RA and to determine the T cell epitopes for this RA.

Figure 2. Comparison (schematic) of the deduced primary amino acid sequences of the full length cDNA clones KBG31, KBG60, and KBG41 and of the C-terminal partial clone KBG7.2. Briefly, mRNA was isolated from the KBG pollen and then a cDNA library was constructed with this mRNA in the expression vector. Screening of this library with a pool of six sera from allergic patients, led to the isolation of cDNA clones. Three homologous full length cDNA clones, KBG 31, 41 and 60, encoding a major group of KBG pollen allergens were reported. These clones exhibit an exceptionally high degree of similarity among them but had no homology with other known proteins or genes. KBG7.2 corresponded to the C-terminal of the full length cDNA clones and was further analyzed.

Each *differently shaded domains* represents a sequence present in two or more of the molecules. *Blank domains* represent non-homologous sequences. The *arrows* represent single amino acid substitutions.

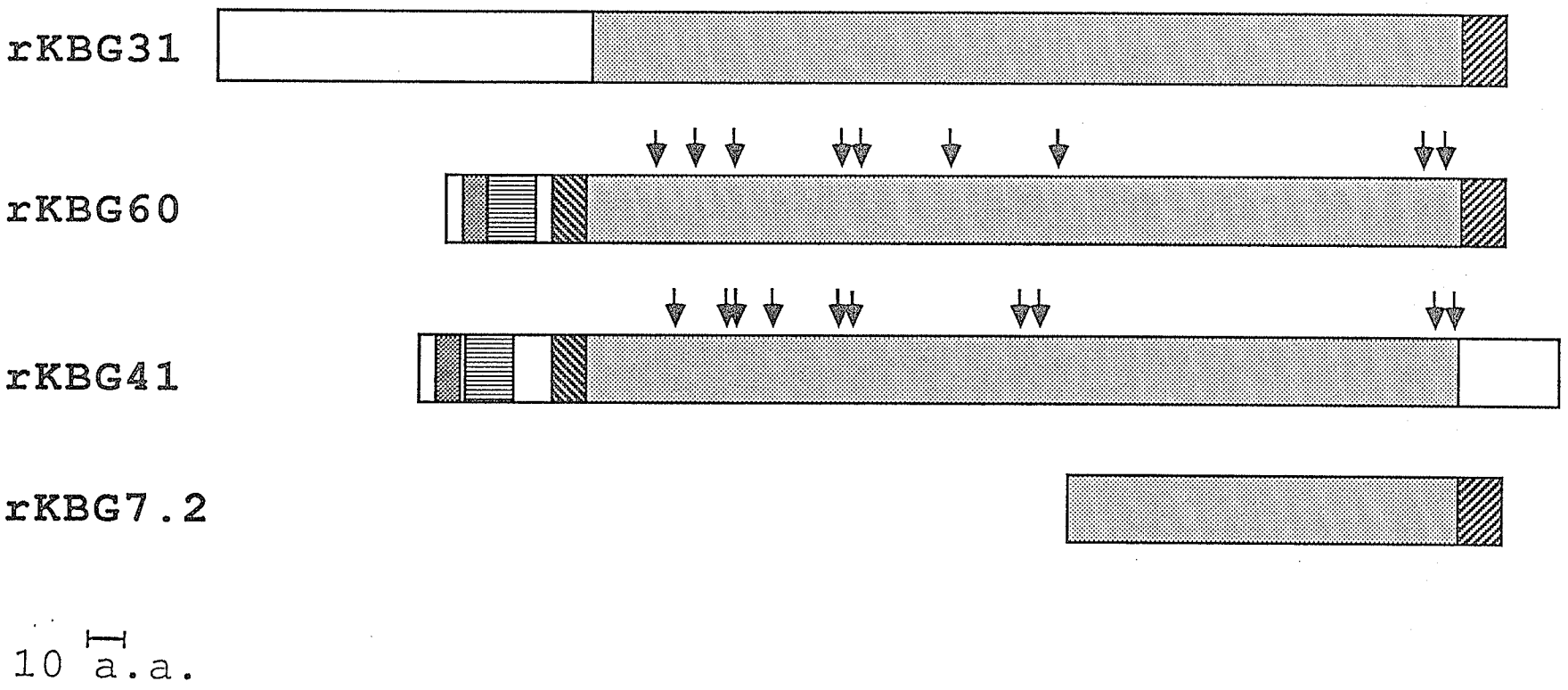



Fig. 2

## III. MATERIAL AND METHODS

### Human sera

Sera were obtained from 55 individuals attending Chedoke-McMaster Hospital who exhibited positive skin tests for grass-pollen allergens (Kindly provided by Dr. Jerry Dolovich). Sera of non-selected allergic patients (n=978) were obtained from the International HLA-Allergy Study conducted under the auspices of the 11th International Histocompatibility Workshops and Conferences hold in Yokohama, Japan in November 1991.

### Skin tests

The grass-mix skin test allergen contained redtop, June grass, orchard, and timothy grass (Bencard, Mississauga, Ontario, Canada). The epicutaneous SPTs were performed on the forearm with a 26-gauge hypodermic needle with the consent of the subject. Grading was as follows: (i) no wheal, negative (-); (ii) 1 to 2 mm wheal, (+); (iii) 3 to 5 mm wheal, (++); (iv) 6 to 9 mm wheal, (+++); and (v) >9 mm wheal, (++++). Histamine and saline were used as positive and negative controls. The skin tests were read after 15 minutes (skin tests were done by Mrs. Magon O'Conner in Hamilton, Ontario, Canada).

Grass pollens

Pollens of the following 10 different species of grasses were obtained from Hollister-Stier Laboratory, Mississauga, Ontario: KBG (*Poa pratensis*), ryegrass (*Lolium perenne*), timothy (*Phleum pratense*), Johnson (*Sorghum halepense*), quack (*Agropyron repens*), tall oat (*Arrhenatherum elatins*), smooth brome (*Bromus inermis*), red top (*Agrostis maritima*), Canada blue (*Poa compressa*), and orchard (*Dactylis glomerata*).

Protein isolation from grass pollens

A 1% (wt./vol.) suspension of pollens in TBS with 1 mmol/L of phenylmethylsulfonyl fluoride was incubated with shaking overnight at 4 °C. The debris was removed by filtration and centrifugation, and the supernatant containing pollen proteins was stored in aliquots at -20 °C. A preparation of KBG pollen proteins was used in all comparative experiments. In addition, a gel-purified fraction of KBG pollen proteins consisting of proteins in the range of 27 to 35 kDa, which included the proteins corresponding to the RA and group I proteins (59), was also used in these experiments. The gel-purified fraction of KBG pollens was isolated by separation of the KBG pollen extract in a 10% SDS-polyacrylamide gel according to the method of Laemmli (70) and excision of the gel at the M.W. 27 to 35 kDa. The proteins were eluted from the gel with an electroelution apparatus (Bio-Rad, Rockville Centre, N.Y.). The protein

concentration was determined by Bio-Rad protein assay. The protein preparation was sterilized by filtration through 0.22 $\mu$m membrane and was stored at -20 °C.

Expression of KBG7.2 in pWR590 plasmid*

The plasmids of pW590 series include the *E. coli lac* promoter and a portion of the coding sequence of $\beta$-galactosidase, which could code for approximately 590 or 450 amino acids was chosen for the expression of KBG7.2 and the construction of expression vectors for the synthesis of fusion proteins is shown in Figure 3 (71). Based on the sequence data of KBG7.2, pWR590.1 was chosen for the expression of the allergenic peptide encoded by KBG7.2 (68). The plasmid pWR590.1 was digested with *Eco* RI, precipitated with ethanol, and then treated with bacterial alkaline phosphatase. The KBG7.2 insert was prepared by *Eco* RI digestion and isolated with "gene clean" procedures (Bio Can Scientific, Mississauga, Ontario). The insert was ligated to the pWR590.1 and transformed in *E. coli* strain JM105. Ampicillin-resistant transformant colonies were screen for expression of the FP.

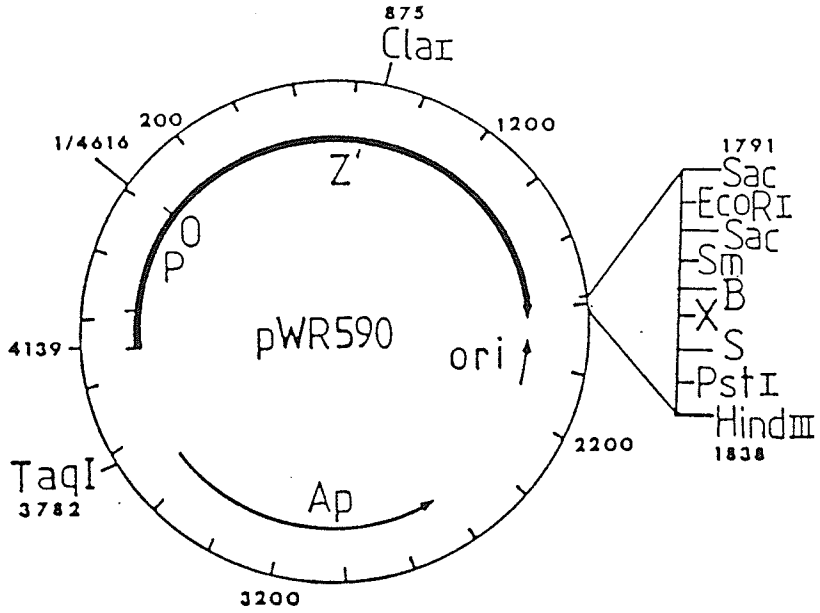
---

* The expression of KBG7.2 in pWR590 plasmid was prepared and kindly provided by Egil Olsen.

Figure 3. Construction of expression vectors for the synthesis of fusion proteins

Physical map of pW590 which contains the *lac* promotor (p), operator (o) and the coding sequence for approximately 590 amino acids of *lac Z*. Large amounts of fused proteins can be produced by ligating any suitable gene to the polylinker region at the 3' end of pW590.

A




B

| | | |
|---|---|---|
| | | 582 583 |
| | | Gly Asn |
| pWR590 | GGC AAC CGG GCG AGC TCG$^{+}$AAT TCG | |
| | | 582 583 |
| | | Gly Asn |
| pWR590-1 | GGC AAC CCG GGC GAG CTC G$^{+}$AA TTC | |
| | | 598 599 |
| | | Asp Arg |
| pWR590-2 | GAT CGC GGG CGA GCT CG$^{+}$A ATT CGA | |

C

| | | |
|---|---|---|
| | β-galactosidase | 1007 proinsulin |
| pSI-1007 | CCG TCA GTA TCG GCG GAA TCC CGG ATG$^{+}$TTT GTC | |
| | | 583 |
| pWR590-BCA4 | AAC CCG GGC GAG CTC GAA TTC CGG ATG$^{+}$TTT GTC | |

Fig. 3

Purification of the RA

One litre culture of the IgE binding transformants, that is , carrying the recombinant plasmid, was grown overnight in Luria broth containing 100$\mu$g/ml of ampicillin at 37 °C without any inducer. Cells were harvested by centrifugation and resuspended in 60 mM tris-HCI pH 6.8, 2% (w/v) SDS, 5% 2-mercaptoethanol, 3 M urea. The intact cells were boiled for 5 minutes and separated by SDS-PAGE on a 10% gel (72). The RA band was excised from the gel, and the protein was eluted from the polyacrylmide gel; the appropriate gel slice being crushed and shaken for 16 hours at 37 °C in a buffer containing 50 mmol/L of Tris-HCL, pH 8.0, 1mmol/L of CaCl, and 0.1% (wt/vol) of SDS that solubilized the RP (73). This preparation was filter sterilized before use. With this procedure from 1 L of culture, it was possible to obtain about 10 mg of RA.

SDS-PAGE

SDS-PAGE was performed in a discontinuous system according to Laemmli (74) in a Bio-Rad mini slab gel apparatus. Samples were prepared in sample buffer and boiled for 5 minutes. The gel was stained with Coomassie Brilliant blue. Molecular weight pre-stain protein standards (BRL) were used to calibrate the gels for determination of the relative molecular weights of the electrophoresed components.

Western immunoblot

Proteins separated by 10% SDS-PAGE were electrophoretically transferred onto nitrocellulose membrane (75). The membrane was blocked by incubation with 1% BSA/PBS for at least 3 hours and then incubated with 1:10 diluted human serum at room temperature overnight. Ab binding was detected by incubation with 1:1000 diluted alkaline phosphatase-conjugated goat anti-human IgE (Tago Inc., Burlingame, CA) for 2 hours. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used as substrates. The membranes were washed 4 times for 5 minutes with PBS/0.05% Tween 20 between each step.

ELISA

The protein was coated onto microtiter plates (Costar, Mississauga, Ontario) in a buffer, containing 0.01 mol/L of $NaCO_3$ and 0.01 mol/L of $NaHCO_3$ (pH 9.6) at 4 °C overnight, blocked by 1% BSA in PBS for another 3 hours, and then incubated with human sera at a dilution of 1:10 for a minimum of 6 hours. The bound IgE was washed and incubated with alkaline phosphatase-conjugated goat anti-human IgE (Taco Inc.) at 1:1000 dilution in PBS with 0.1% BSA and 0.05% Teen 20 for 3 hours. For the detection of the Ab reaction in ELISA, p-nitrophenyl phosphate was used as a substrate, and the O.D. was measured at 405 mm by an ELISA reader. Each incubation step was followed by washing with 0.05% Tween 20 in PBS. The ELISA background was measured by use of a pool of sera of five

non-atopic individuals and each ELISA experiment was repeated twice.

ELISA inhibition

96 well microtiter plates were coated with KBG pollen extract or RA at the concentration of 30 $\mu$g/ml and incubated at 4 °C for overnight. Sera diluted 1:10 to be tested were pre-incubated with KBG pollen extract or RA 30 $\mu$g/ml at room temperature for 3-5 hours. In the control experiment, diluted sera were pre-incubated with PBS. Following this, 100 $\mu$l sera pre-incubated with Ag were added to the wells coated with antigen. After 6 hours of incubation and three additional washes, 100$\mu$l diluted 1:1000 goat anti-human IgE was added, then p-nitrophenyl phosphate was used as a substrate. The O.D. was measured in an ELISA reader. The percentage of inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = \left(1 - \frac{\text{O.D. with inhibitor}}{\text{O.D. without inhibitor}}\right) \times 100$$

Immunization

Female C3H/HeJ mice, 6-8 weeks old were immunized in the hind foot pads with either KBG pollen extract or RA (60 $\mu$g per mouse) emulsified with Freund's complete adjuvant (Sigma) according to the procedure of Lee *et al.* (76). Two

weeks after immunization, the mice were sacrificed to obtain the popliteal lymph nodes.

Lymphnode cells proliferation assay

Proliferation of cells in the presence of Ag was carried out as described by Ekramoddoullah *et al.* (77). A suspension of single cells was prepared from popliteal lymph nodes (PLN) in RPMI-1640 (Gibco), supplemented with 2 mmol/L of L-glutamine, 5 x $10^{-5}$ mM 2-mercaptoethanol, 100 u/ml of penicillin G, 100 $\mu$g/ml of streptomycin (Sigma), and 10 % heated inactivated fetal bovine serum (Gibco) at a concentration of 5 x $10^6$/ml. One hundred microliters of this cell suspension ($5x10^5$ cells) was then pipetted into each flat-bottom well of tissue culture plate containing 96 wells . Ags were diluted to the appropriate concentration with complete culture media. One hundred microliters of each antigen concentration was pipetted into each well. For control, 100$\mu$l culture media was added to a well containing the cells. For each antigen concentration, cell cultures were carried out in duplicate. Cells were cultured for 3 days at 37 °C in the incubator with 5% $CO_2$. [$^3$H]-thymidine was added at 1 $\mu$Ci per well during the last 16 hours of culture. The cells were then harvested onto glass fibre filters (Flow Laboratories). Each filter disc was put into a scintillation vial to which 2 ml of scintillation fluid was added and then counted in a $\beta$-counter (Beckman Liquid Scintillation Counter).

Preparation of PBMCs

20 ml of venous blood was collected into sterile plastic syringes containing 250 u/ml of heparin, diluted 1:2 with balanced solution in 50 ml sterile centrifuge tubes and underlayed with Ficoll-Hypaqe (Pharmacia) in a ratio of 1:3 according to the instructor from pharmacia Co.. The tubes were centrifuged at 400 g for 30 minutes. Interphase cells were removed and washed three times, twice with balanced salt solution, and once with complete culture medium. These cells are subsequently referred to as PBMCs.

EB-Virus preparation

EB-Virus preparation was carried out according to the technique described by E. V. Walls and D. H. Crawford (78). Briefly, B95-8 cells (Kindly provided by Dr.E. Berczi) were grown in plastic tissue flasks at concentration 1 X $10^5$ cells/ml in a 37 °C incubator. When the cells reached a density of 5 X $10^5$/ml they were left for about 10-14 days without replacing the medium. Following culturing, the culture supernatant was harvested and centrifuged at 400g for 15 minutes at 20 °C to remove most of the cells, then passed through a 0.45 $\mu$m pore filter. The supernatant containing EBV stocks were stored in -70 °C.

Establishment of EBV immortalized cell lines

PBMCs pellets were resuspended up to $10^7$ cells in 1 ml of undiluted B95-8

culture supernatant and incubated at 37 °C for 1 hour in a 5% $CO_2$ incubator, agitating the cells occasionally to keep them in suspension. Following incubation with EBV, cells were resuspended in complete medium at 1 x $10^6$/ml and 2 ml aliquots were dispensed to 24 well flat-bottom tissue culture plates. Cyclosporin A was added to the culture medium to a final concentration of 0.1-1 $\mu$g/ml. At weekly intervals, half of the supernatant was removed from the culture it and replaced with fresh medium (78). After 3 weeks of culture, supernatant was harvested and the antibody production was measured by ELISA.

Preparation of Ag specific T cell lines and clones

For the production of T cell lines and clones, the technique described by P. M. Taylor *et. al.* (79) was used with appropriate modifications as described below. PBMCs prepared from atopic patients' blood were cultured at 2-4 X $10^6$/ml in 24 well culture plate with KBG pollen extract 50 $\mu$g/ml for one week. Then APCs (EBV-transformed autologous B cells) were irradiated 10,000 rads. and added to the culture at a final concentration 5 X $10^5$/ml. At the same time, KBG pollen extract 50 $\mu$g/ml and rIL-2 10u/ml was also added. The cells cultured for another two weeks. For cloning, the limiting dilution cloning method described previously was used (80). Briefly viable cells 1 cell/well were resuspended in complete medium and plated in 96 well trays together with Ag, autologous EBV transformed B cells (irradiated 10,000 rads) and rIL-2. Thereafter, Th lines and clones were

maintained by feeding with Ag, APC and rIL-2 every 7-10 days. Before using the cells for proliferation assays the cells were rested for 5-7 days after the last addition of filler cells and Ag.

T cell proliferation assay

Proliferation assays were performed in 96-well microtiter plate. The PBMCs were resuspended to a concentration of 5 X $10^6$/ml in complete medium with 5% human AB+ serum. PBMCs (5 X $10^5$/well) were cultured in the absence or presence of antigen or peptide. Five days later [$^3$H]-thymidine was added and its incorporation into DNA was measured after 16-18 hours. When T cell lines or clones were used, autologous EBV-transformed B cells (as a source of APC) which pre-incubated with appropriate Ag or peptide at 37 °C incubator for 2-4 hours, then incubated with mitomycin C (1 -2 $\mu$g/ml) for 1 hour and washed for four times. Prior to use for T cell proliferation studies T cell blast (1 X $10^5$/well) and 0.5 X $10^5$/well of irradiated (10,000 rads) B cells (pre-treated) were cultured for 3 days, [$^3$H]-thymidine was incorporated during the last 16-18 hours of culture. The values given are the arithmetic mean cpm of triplicate cultures .

Cell markers

T cells were examined for CD3, CD4, CD8, and Ia expression by two colour flow microfluorometric analysis. 1 X $10^6$ cloned T cells are suspended in RPMI

with 15% FCS and incubated with CD4-FITC vs CD8-Rhodamine and CD3-FITC vs Ia-Rodamine for 40 minutes at 4 °C. After removing of excess antibody, 8% formalin was added to the cell pellet and mixed. The flow cytometer on cells was gated with the scatter characteristics of lymphocytes. 10,000 cells were counted and the results were expressed on the basis of two-colour histograms as % CD4-FITC positive cells (green; X) vs % CD8-Rhodamine positive cells (red; Y) and % CD3-FITC positive cells (green; X) vs % DR-Rhodamine positive cells (red; Y) within the cell population.

In this study, the above mentioned protocols were employed to achieve the three major goals as studied before. For the purification of RA, the bacterial proteins were separated by SDS-PAGE and western blotting. The allergenic properties were assayed by ELISA. The ability of RA to recognize lymphocytes were assayed by lymphnode proliferation assay in murine model, and by proliferation of human PBMCs and T cell lines and clones.

# IV. RESULTS

## (A) IgE-BINDING STUDIES OF RECOMBINANT ALLERGEN

### (i) Synthesis and purification of recombinant allergen

For identification of the polypeptides encoded by cDNA clone, KBG7.2, which comprised sequences common to all three clones described earlier, was cloned into an expression plasmid which permitted high level expression of the corresponding recombinant peptide (KBG7.2). A Western immunoblot employing a sera pool of grass pollen allergic patients to KBG extract is shown in Fig. 4. It is clear that in the KBG pollen extract the IgE antibodies in allergic patients' sera pool recognized several protein bands with estimated molecular masses of 28 to 34 kDa, 35 kDa and 57 kDa. In Fig. 5 is illustrated an electrophoretogram FP-producing *E.coli* extract separated by SDS-PAGE (c) and purified FP by cutting the 81 kDa band on SDS-PAGE of RA-producing *E.coli* extract (d). The RA appeared as a major 81 kDa FP band in electrophoretogram of RA-producing *E.coli* extract but was absent in control *E. coli* extract i.e. bacteris with self-ligated plasmid (Fig. 5, b). A Western immunoblot of the above gel was treated with same sera pool for detection of IgE-binding proteins. The IgE antibodies in this pool of sera reacted strongly with the 81 kDa RA (Fig.6, a). No Ab binding was observed for the

Figure 4. SDS-PAGE and Western immunoblot of KBG pollen extract

a: Protein molecule weight makers.

b: SDS-PAGE of KBG pollen extract with Coomassie Brilliant blue.

c: Western immunoblot analysis of KBG extract. The immunoblot was developed with a sera pool from grass pollen allergic patients.

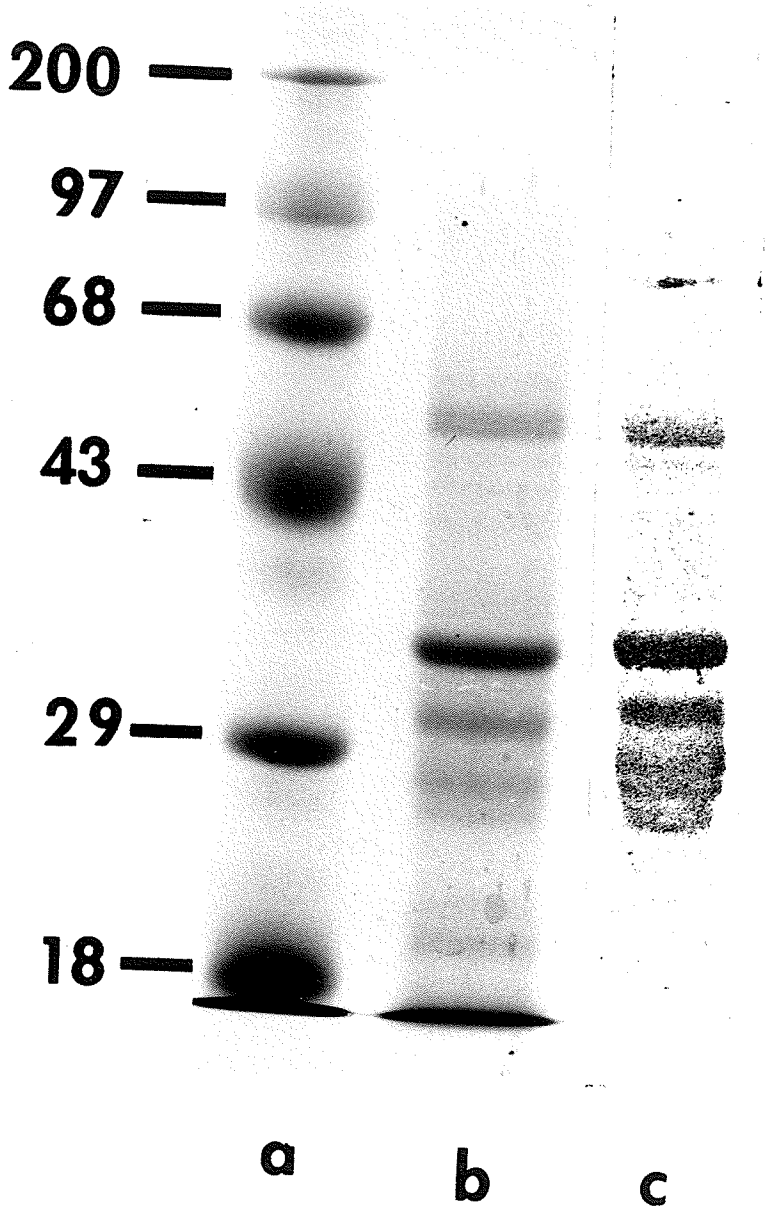



Fig. 4 - P. 43a

Figure 5. SDS-PAGE of recombinant allergen

a: Protein molecule weight makers.

b: SDS-PAGE of *E.coli* extract without KBG7.2 insert.

c: RA-producing *E.coli* extract separated by SDS-PAGE with Coomassie Brilliant blue.

d: SDS-PAGE of RA purified by cutting 81 kDa band on SDS-PAGE of RA-producing *E.coli* extract. Briefly, the intact cells carrying the recombinant plasmid were boiled for 5 minutes and separated by SDS-PAGE on a 10% gel. The 81 kDa FP band was excised from the gel, and the protein was eluted from the polyacrylmide gel. The arrows indicate the cutting places.

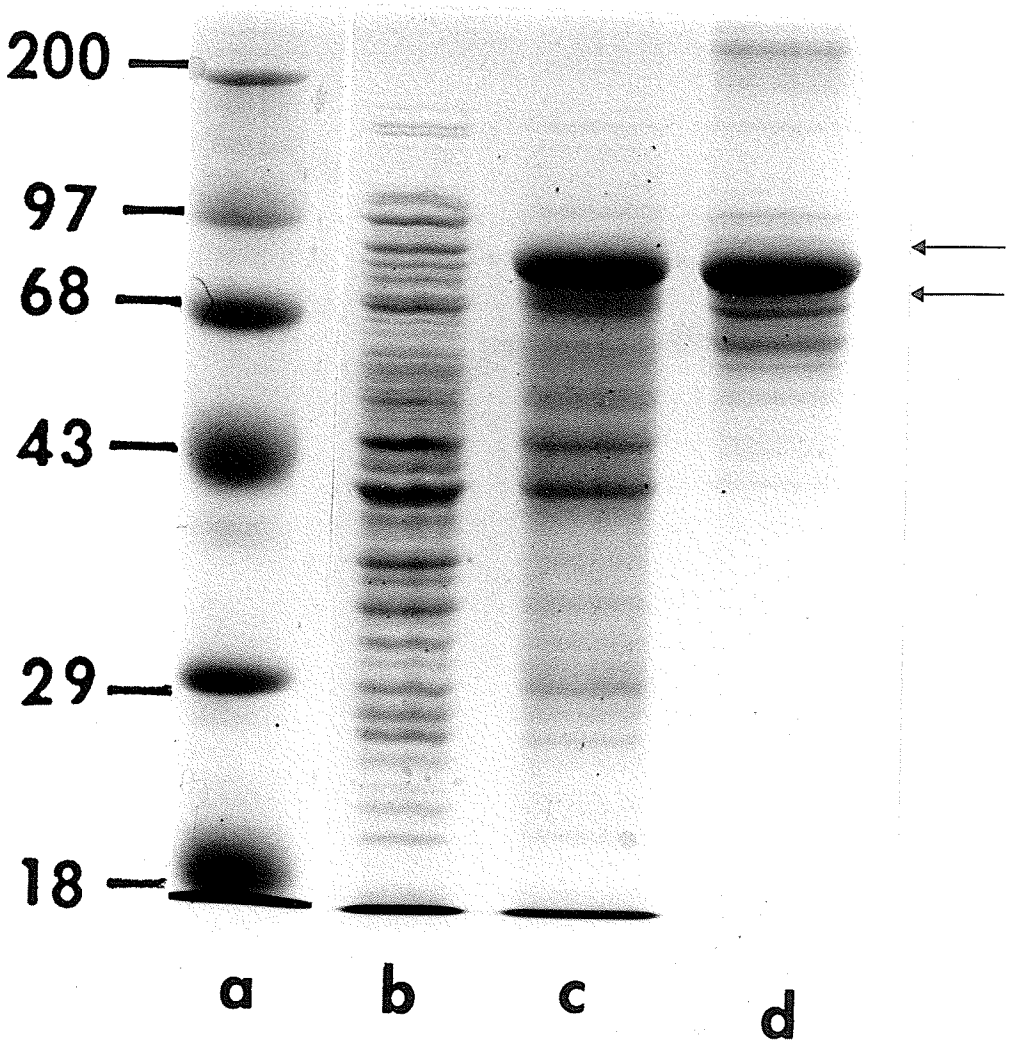



Fig. 5 - P. 44a

Figure 6. Western immunoblot of recombinant allergen

a: Western immunoblot analysis of RA-producing *E.coli* extract. *E.coli* extract was separated by SDS-PAGE and transferred to nitrocellulose membrane which was developed with a sera pool from grass pollen allergic patients.

b: Western immunoblot of control *E.coli* extract. No IgE binding was observed.

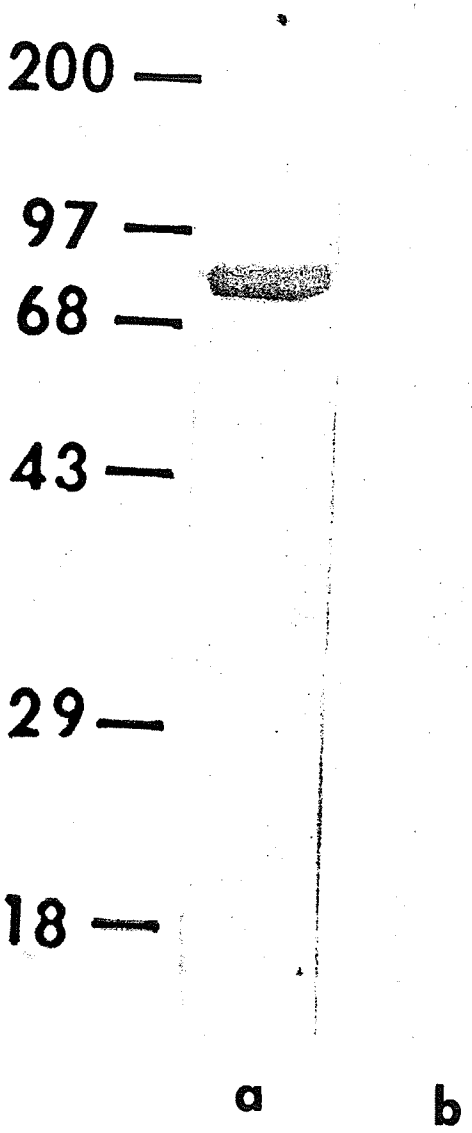



Fig. 6 - P.45a

proteins isolated from control *E.coli* extract (Fig. 6, b). This method provided >95% pure recombinant allergenic proteins and as showed in Fig.5, the contaminating proteins <5% were non-allergenic proteins that they did not bind to human IgE antibodies.

(ii) Development of ELISA procedure for detection of specific IgE binding of RA

ELISA procedure was employed to test specific IgE antibodies to KBG pollen extract and RA in the sera of allergic patients. Specific IgE antibodies to KBG and RA in serial dilutions of a pool of sera from five grass pollen allergic patients were measured by ELISA. A comparable dilution curve for KBG and RA was shown in Fig. 7. The flat curves were seen in serum dilution 1:2 to 1:10. After that, as serum dilution increased, the curves decreased until O.D. = 0. This result indicated that 1:10 is the optimal serum dilution for testing specific IgE Ab to KBG as well as RA. In the following studies, all sera were tested at 1:10 dilution.

Reactivity of KBG and RA with specific human IgE in allergic patients' sera was assessed by ELISA inhibition system. As shown in Fig. 8 (a and b), a serial dilution of inhibitors was used, with BSA as a negative control; almost 100% of inhibition was obtained by KBG and RA to respective antigens at inhibitor concentration 100 $\mu$g/ml and no inhibition was seen by BSA at any concentration. Five individual sera showing high IgE levels were also used in inhibition assay. Fig. 9 ( a, b ) indicated sera preincubated with RA inhibited 50-70% of specific

Figure 7. Serum dilution curves of specific IgE to KBG and RA

Specific IgE to KBG and RA in a serial dilution of a allergic patient's serum were measured by ELISA. KBG and RA were coated to the plate at the concentration of 10 $\mu$g/ml. Serum dilution of 1:10 gave the highest O.D. value and was considered optimal for testing specific IgE Ab to KBG as well as RA.

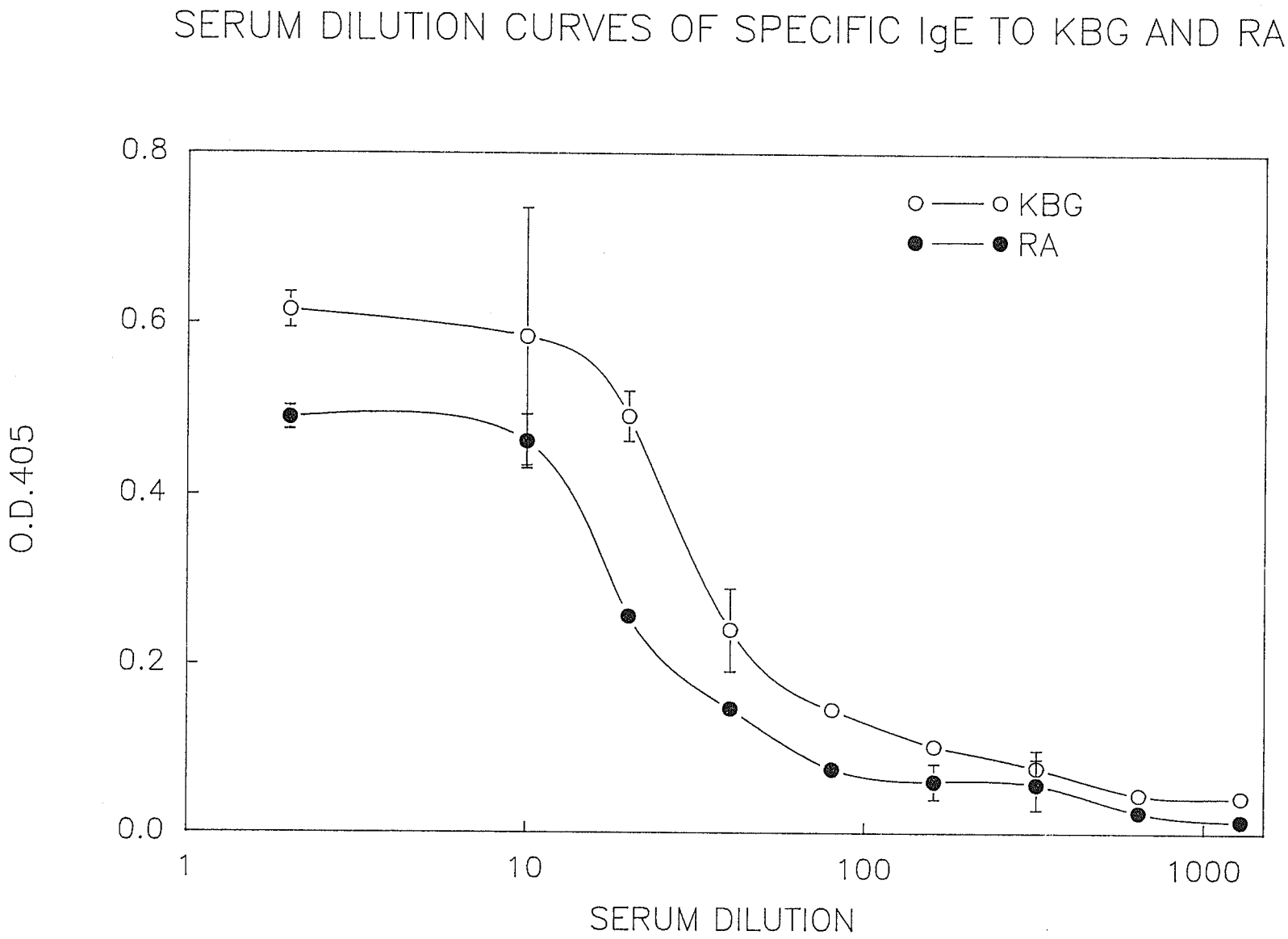

SERUM DILUTION CURVES OF SPECIFIC IgE TO KBG AND RA
KBG
RA
O.D.405
0.8
0.6
0.4
0.2
0.0
1
10
100
1000
SERUM DILUTION

Fig. 7

Figure 8. ( a, b ) Inhibitor dilution curves

A sera pool of allergic patients (1:10 dilution) was pre-incubated with a serial dilution of RA (a) or KBG (b) extract and added to the plates which were coated with RA or KBG (10$\mu$g/ml). BSA, an unrelated antigen, in same dilutions was also used as control for inhibitor. The extent of binding of human IgE to RA or KBG was evaluated by addition of AP-conjugated mouse monoclonal anti-human IgE antibody.

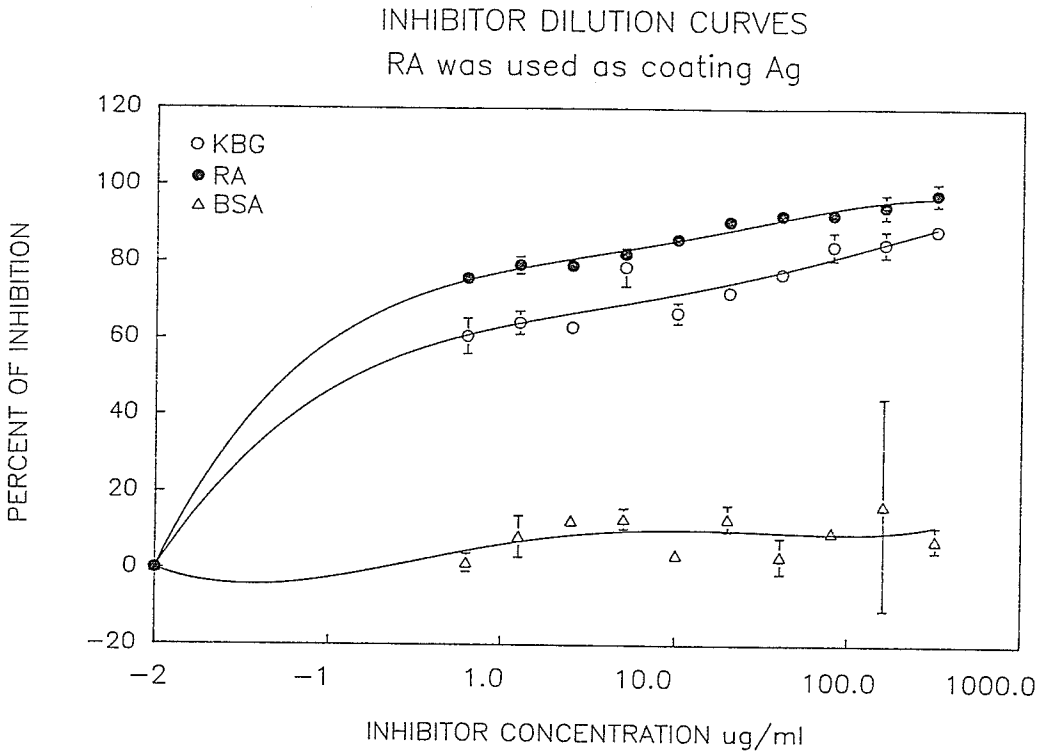

INHIBITOR DILUTION CURVES
RA was used as coating Ag
KBG
RA
BSA
PERCENT OF INHIBITION
120
100
80
60
40
20
0
-20
-2
-1
1.0
10.0
100.0
1000.0
INHIBITOR CONCENTRATION ug/ml

Fig. 8 (a)

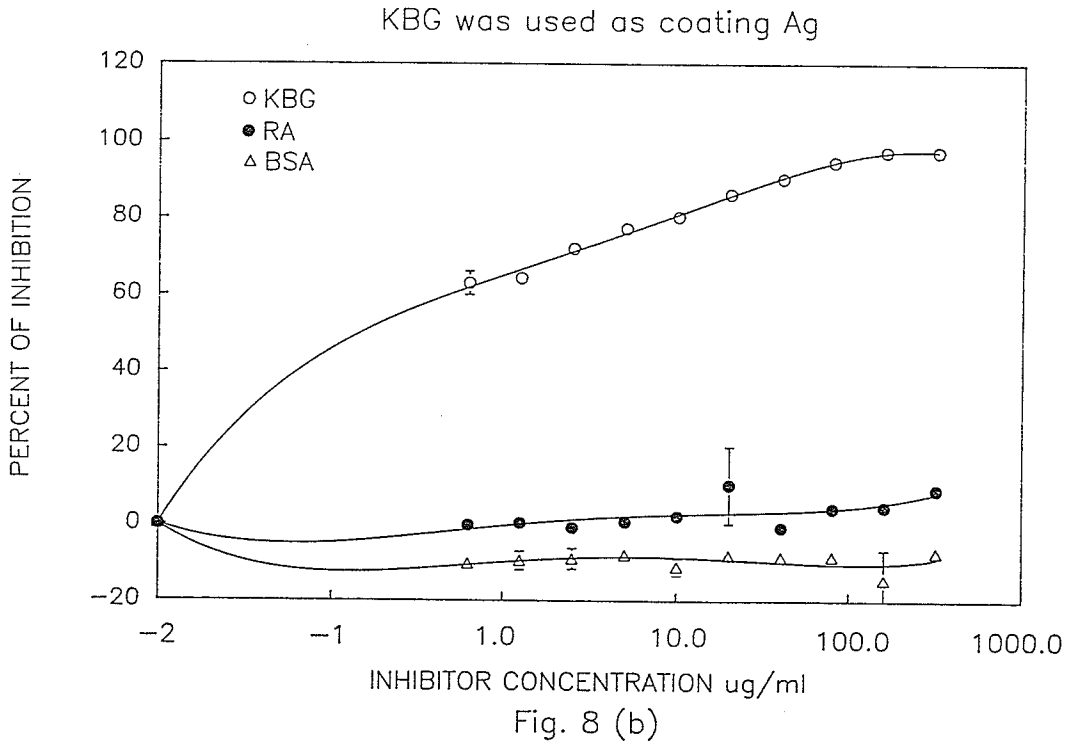

KBG was used as coating Ag
KBG
RA
BSA
PERCENT OF INHIBITION
120
100
80
60
40
20
0
-20
-2
-1
1.0
10.0
100.0
1000.0
INHIBITOR CONCENTRATION ug/ml

Fig. 8 (b)

Figure 9. ( a, b ) ELISA inhibition assay by individual serum

Five individual sera were pre-incubated with either RA (a) or KBG (b) at the concentration of 10$\mu$g/ml and added to the plates which were coated with RA or KBG (10$\mu$g/ml). The percent of inhibition was shown in this figure. This is demonstrated that IgE binding to KBG and RA is specific.

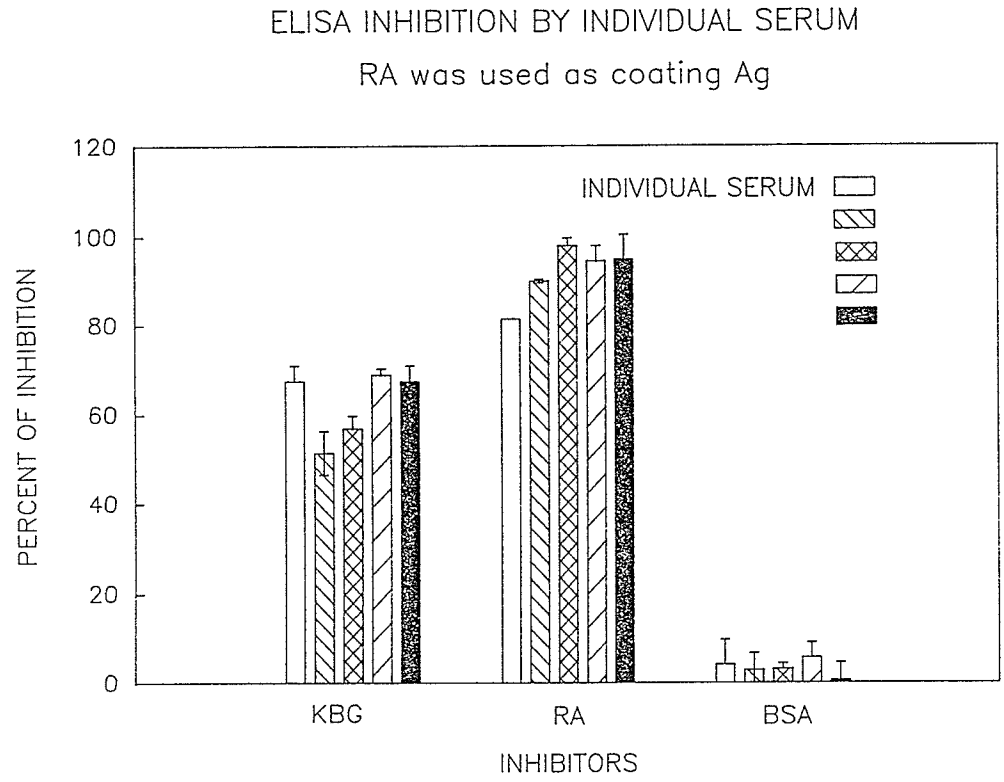

ELISA INHIBITION BY INDIVIDUAL SERUM
RA was used as coating Ag
INDIVIDUAL SERUM
PERCENT OF INHIBITION
120
100
80
60
40
20
0
KBG
RA
BSA
INHIBITORS

Fig. 9 ( a )

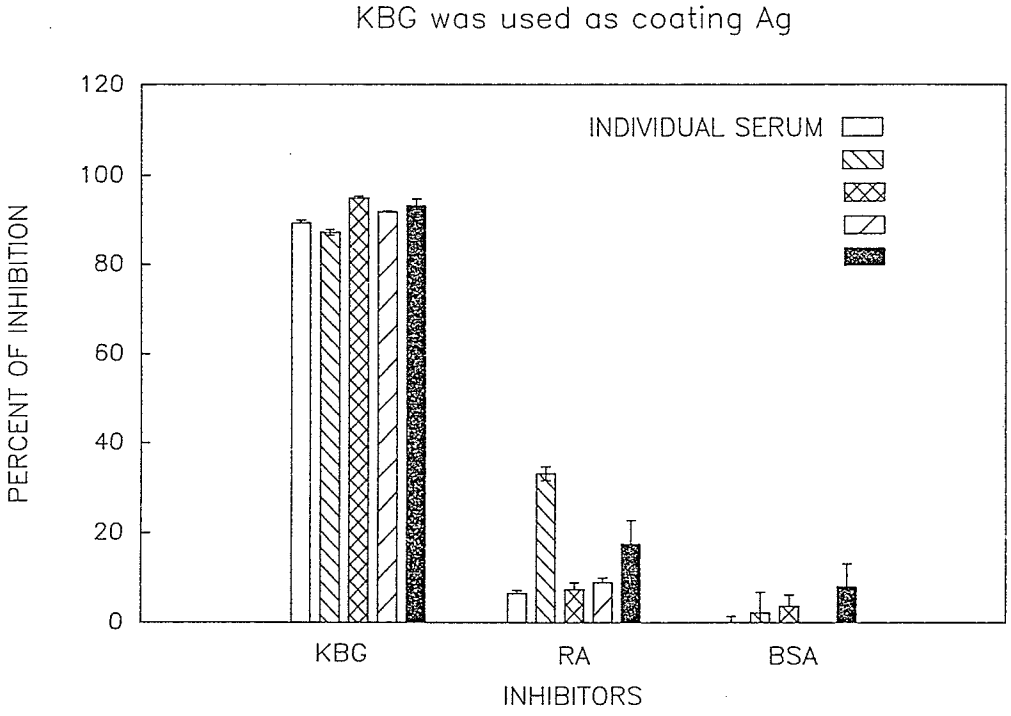

KBG was used as coating Ag
INDIVIDUAL SERUM
PERCENT OF INHIBITION
120
100
80
60
40
20
0
KBG
RA
BSA
INHIBITORS

Fig. 9 ( b )

IgE binding to KBG and 80-95% of binding to RA. However IgE binding to KBG and RA was inhibited 90-95% and 10-40% by the sera which were preincubated with KBG. Similarly, BSA was used as a negative control and did not show any inhibition to either KBG or RA. These results demonstrated that IgE Ab binding to KBG and RA is specific and that ELISA procedure can be used for the assay of IgE-binding properties of RA.

The results from the microtiter ELISA for IgE Ab levels, specific to the KBG, KBG group 1 (which is corresponded to 35 kDa band of KBG extract on SDS-PAGE), and RA are illustrated in Fig. 10. The individual patient's sera demonstrated different IgE Ab levels, and the Ab level varied with different coating Ags. The highest O.D. values were observed with KBG pollen extract, followed by KBG group 1, and then the RA. For each individual serum, the specific IgE level to RA comprised about 50% to 80% (Fig. 9) of that to KBG pollen extract and KBG group 1 . However, sera from five control, nonatopic individual did not demonstrate any detectable reactivity. Similarly, unrelated Ags, such as $\beta$-galactosidase and BSA, did not elicit any IgE binding reactivity with these five sera from allergic patients (data not shown). It is inferred that RA contains a dominant allergenic epitope of grass pollens.

(iii) Frequency of recognition of RA by allergic individuals

To assess the allergenicity of the recombinant peptide, the capacity of RA binding IgE Abs in the sera of allergic individuals was examined. A comparison of the IgE Ab levels of 55 individual sera at 1:10 dilution with KBG pollen extract and with that of RA is illustrated in Fig. 11. A total of 55 allergic patients to grass pollen were classified into three groups based on their SPT results to grass pollen mix; that is, the sera of patients with a score of (++), (+++), or (++++) were examined separately. Clearly high scores in SPT were associated with high specific IgE levels, irrespective of the Ag, KBG pollen extract or RA. These results indicated that within a given group of patients with the same SPT scores, the relative IgE binding to the RA was comparable to that of natural KBG pollen. Furthermore, a high SPT score was associated with high specific IgE binding.

Since the RA reacted with >90% of grass pollen allergenic patients' sera examined, which suggested that the cloned peptide is a clinically relevant allergenic component of grass pollen, we compared the IgE Ab reactivity of 31 individual sera of RA with that of KBG or even mixture of proteins from 10 different grass pollens. A positive correlation ($r=0.90$, $p<0.05$) was found between the IgE levels in individual sera specific for RA and KBG (Fig. 12, b). Similarly, a positive association was observed in the levels of IgE Abs corresponding to RA and mixture of 10 grass-pollen extracts ($r = 0.90$, $p < 0.05$)

Figure 10. Comparison of specific IgE reactivity to different Ags

Comparison of specific IgE reactivity in five allergic patients (indicated by symbols) and five non-atopic individuals as control (indicated by solid bars) by ELISA. A concentration of 10$\mu$g/ml for all antigens was used to coat ELISA plates.

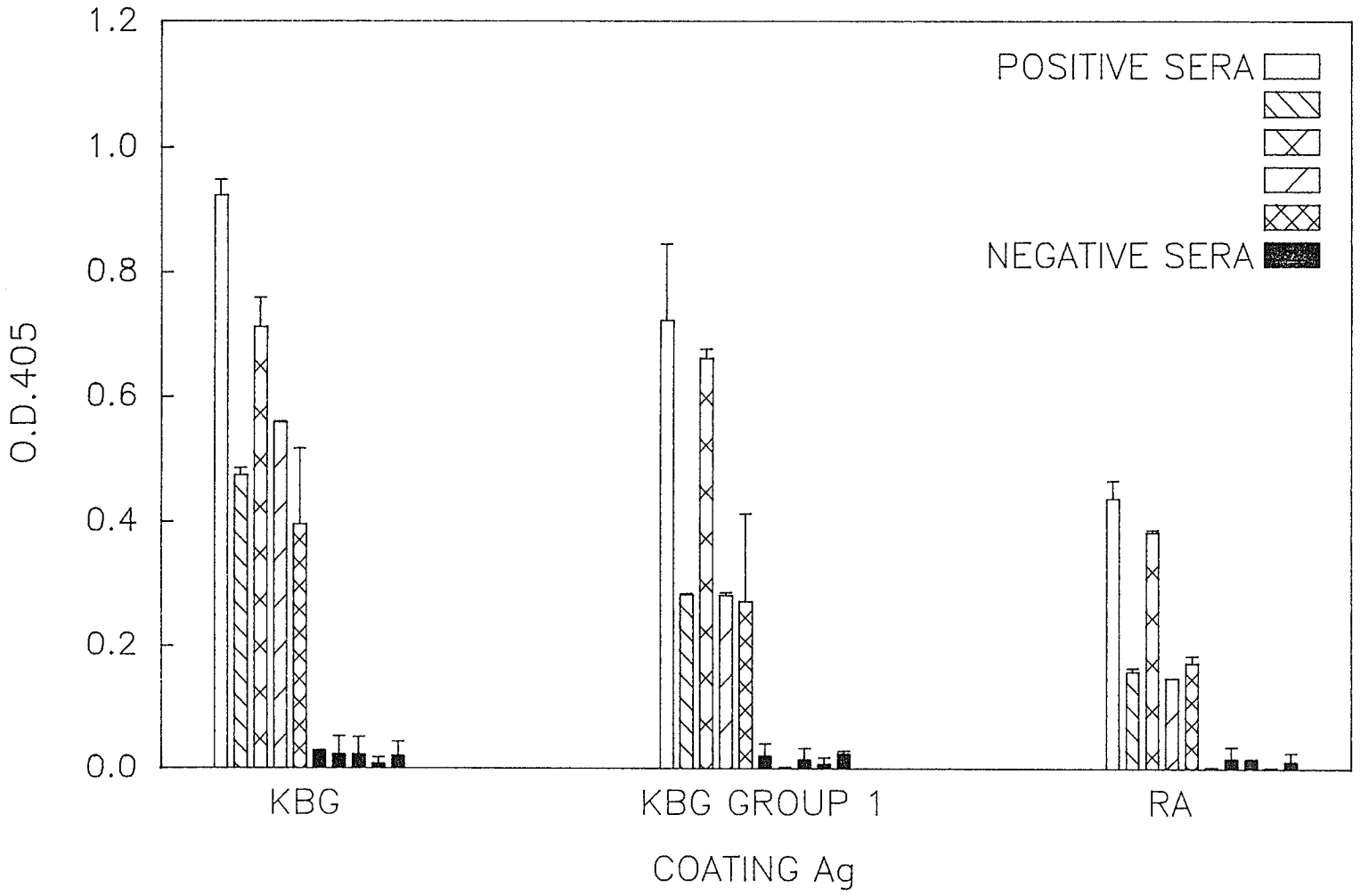

COMPARISON OF SPECIFIC IgE REACTIVITY
TO DIFFERENT AgS
1.2
1.0
0.8
0.6
0.4
0.2
0.0
O.D.405
POSITIVE SERA
NEGATIVE SERA
KBG
KBG GROUP 1
RA
COATING Ag

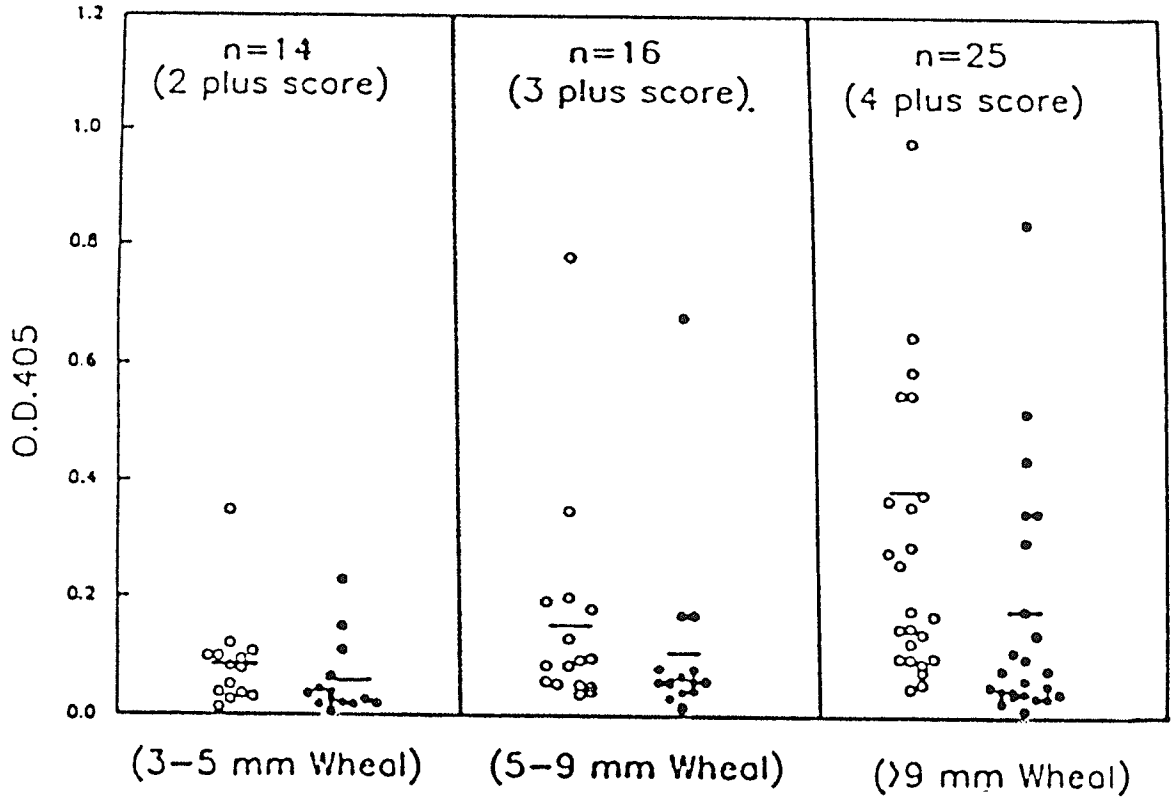
Fig.10

Figure 11. IgE reactivity of sera of patients' with distinct clinical symptoms to RA or KBG by ELISA

A total of 55 allergic patients to grass pollen were classified into three groups based on their SPT results to grass pollen mix. Clearly high scores in SPT were associated with high specific IgE levels.

The individual O.D. represents the value after subtraction of the background. The background was measured by use of a pool of five sera (as negative control) of individuals who were not allergic to grass pollens, as measured by SPTs. No IgE reactivity was detectable by immunodot assay.

IgE REACTIVITY OF SERA OF PATIENTS WITH DISTINCT CLINICAL SYMPTOMS TO RA OR KBG BY ELISA




SKIN–PRICK TEST SCORES ( ∘ KBG, • RA )

Fig. 11

Figure 12.(a, b) Correlation analysis of IgE reactivity with RA versus grass pollen mix and KBG

Correlation analysis of IgE reactivity by ELISA with RA versus a protein mixture of ten different grass pollens (pollens of the following 10 different species of grasses were obtained from Hollister-Stier Laboratory, Mississauga, Ontario: KBG (*Poa pratensis*), ryegrass (*Lolium perenne*), timothy (*Phleum pratense*), Johnson (*Sorghum halepense*), quack (*Agropyron repens*), tall oat (*Arrhenatherum elatins*), smooth brome (*Bromus inermis*), red top (*Agrostis maritima*), Canada blue (*Poa compressa*), and orchard (*Dactylis glomerata*)) as coating Ags (a) and of reactivity with RA versus KBG pollen extract as coating Ags (b). Positive correlations in IgE reactivities ($r=0.9$), were observed between RA and KBG as well as between RA and grass mix.

CORRELATION ANALYSIS OF IgE REACTIVITY WITH RA VERSUS GRASS POLLEN MIXTURE

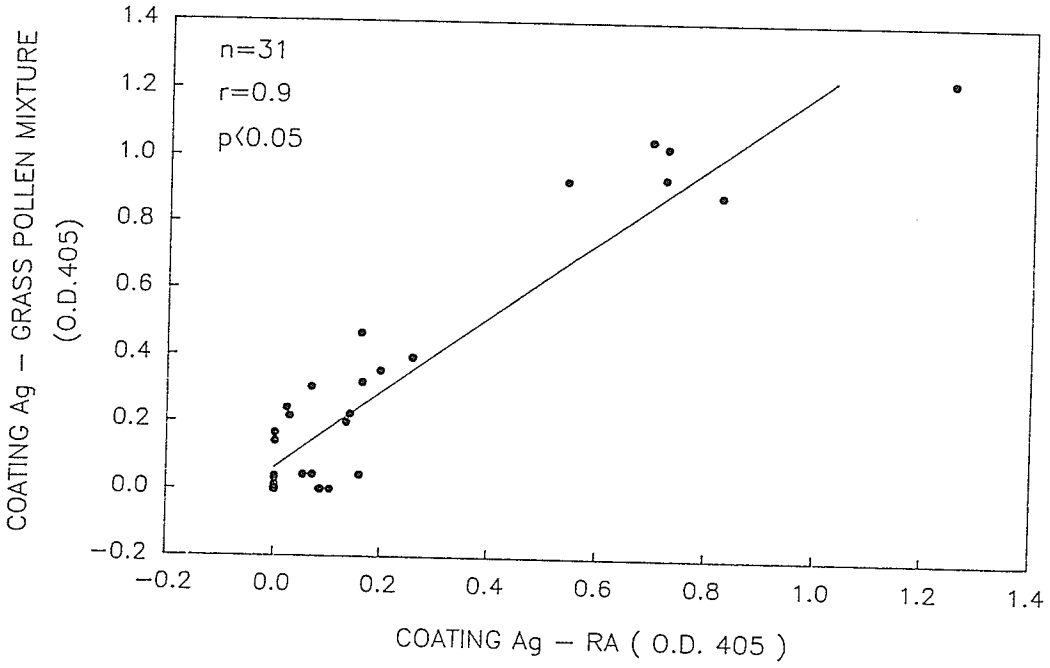



Fig. 12 (a)

RA VERSUS KBG

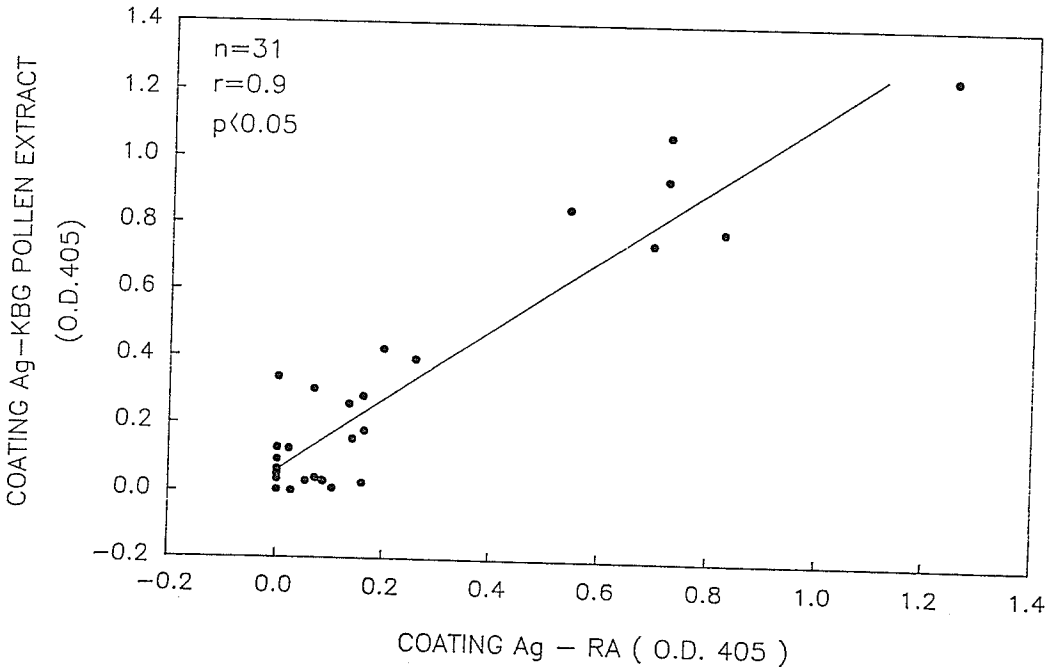



Fig. 12 (b)

(Fig. 12, a). However, the proportion of allergic population recognizing RA and if the RA containing both human IgE and IgG4 binding sites remained unclear.

(iv) IgE and IgG4 antibody binding of RA in non-selected allergic population

We have examined the IgE and IgG4 antibody reactivities to KBG and RA in 978 allergic individuals. 51.1% of total allergic patients' sera showed specific IgE binding to KBG pollen extract, whereas, 39.4% bound to RA. IgE and IgG4 correlation analysis of total allergic patients' sera shown in Table 2, a positive correlation was found in reactivity of IgE and IgG4 antibodies between KBG extract and RA (IgE: $r=0.656$, $P<0.0001$ and IgG4: $r= 0.5875$, $P<0.0001$), respectively. Correlations between KBG IgE and KBG IgG4 were also positive ($r=0.5384$, $P<0.0001$). However, correlation coefficient (r) was lower between specific RA IgE and IgG4 ($r=0.3$, $P<0.0001$). Specific IgE and IgG4 antibodies to KBG and RA correlation analysis of RA positive patients' sera * (384 / 978) showed similar correlations and the correlation coefficients were higher compared to those seen with total allergic patients' sera (Table 3). However, no relationship was found between KBG extract and RA with respect to their specific total IgG antibodies, nor was there any relationship between the specific IgE and IgG

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* Sera with O.D. higher than the mean from five negative sera + 2SD were considered RA positive.

Table 2. IgE and IgG4 correlation analysis of total allergic patients' sera (n=978)

| | Correlation coefficient (r) | Probability (p) |
|---|---|---|
| KBG IgE : RA IgE | 0.656 | < 0.0001 |
| KBG IgG4 : RP IgG4 | 0.5875 | < 0.0001 |
| KBG IgE : KBG IgG4 | 0.5384 | < 0.0001 |
| RA IgE : RA IgG4 | 0.3574 | < 0.0001 |

Table 3. IgE and IgG4 correlation analysis of RA positive patients' sera (n=384)

| | Correlation Coefficient (r) | Probability (p) |
|---|---|---|
| KBG IgE : RA IgE | 0.715 | < 0.0001 |
| KBG IgG4 : RA IgG4 | 0.6967 | < 0.0001 |
| KBG IgE : KBG IgG4 | 0.677 | < 0.0001 |
| RA IgE : RA IgG4 | 0.4835 | < 0.0001 |

antibody reactivities with KBG or RA (data not shown).

These results suggested that RA not only contained human IgE binding sites, but also contained IgG4 binding sites which are similar to that of natural KBG. Whereas specific total IgG Ab reactivities differ for RA and KBG extract and IgG binding sites are different from the IgE and IgG4 binding sites for KBG.

## (B) STUDIES OF T CELL RESPONSES TO RA

### (i) Proliferation of murine lymphonode cells with RA

Previously, it was found that RA was capable of eliciting an immune response in mice. Thus, both IgG (26) and IgE (81) Abs specific to the RA were produced by mice when mice were immunized with the RA. To examine the presence of T cell epitopes on the RA, proliferative responses of PLN cells were examined *in vitro* at different Ag doses, 5, 10 and 20 $\mu$g/ml from mice immunized with RA or KBG in FCA. BSA and $\beta$-galactosidase were used as negative controls and Con A was used as a positive control in this proliferation assay system. Results (Table 4, a and b) indicated that PLN cells, obtained from mice immunized with KBG and RA, gave significant proliferative responses compared to negative control (BSA) *in vitro* culture in the presence of Ag. The proliferative responses were dependent on the amount of Ag used *in the in vitro* culture. Thus, the highest proliferative response was obtained at a concentration of 20 $\mu$g/ml of Ags. Positive

Table: 4 (a and b).

Proliferative response of PLN cells obtained from mice immunized with KBG and RA at a dose 60 $\mu$g/mouse. *In vitro* proliferation of PLN cells stimulated at different concentrations of Ag, that is 5, 10, and 20 $\mu$g/ml. Con A was used as a positive control and BSA as well as $\beta$-galactosidase were used as negative controls. The [$^3$H]-thymidine incorporation was measured as counts per minute. The values are the means of triplicates and with background subtracted.

Table: 4 (a). Proliferative response of PLN cells obtained from mice immunized with KBG

| Ag used in vitro cell culture | Concentration of Ag μg/ml | Incorporation of $^{3}$H-Thymidine | |
|---|---|---|---|
| | | cpm ± SD | Stimulation index |
| Recombinant allergen | 5 | 12390 ± 2365 | 3.6 |
| | 10 | 20400 ± 918 | 5.9 |
| | 20 | 36971 ± 3673 | 10.6 |
| KBG pollen extract | 5 | 11450 ± 615 | 3.3 |
| | 10 | 22270 ± 1094 | 6.4 |
| | 20 | 40450 ± 2543 | 11.6 |
| BSA | 5 | 6906 ± 1574 | 2.0 |
| | 10 | 9914 ± 680 | 2.8 |
| | 20 | 18920 ± 578 | 5.4 |
| β-galactosidase | 5 | 8276 ± 292 | 2.4 |
| | 10 | 11950 ± 436 | 3.4 |
| | 20 | 20640 ± 950 | 5.9 |
| ConA | 5 | 46400 ± 3100 | 13.3 |
| | 10 | 91490 ± 6469 | 26.3 |
| | 20 | 3716 ± 1034 | 1.1 |
| Culture medium | - | 3480 ± 493 | 1.0 |

Table: 4 (b). Proliferative response of PLN cells obtained from mice immunized with RA

| Ag used in vitro cell culture | Concentration of Ag μg/ml | Incorporation of $^{3}$H-Thymidine | |
|---|---|---|---|
| | | cpm ± SD | Stimulation index |
| Recombinant allergen | 5 | 7684 ± 1471 | 1.8 |
| | 10 | 15210 ± 1324 | 3.5 |
| | 20 | 24730 ± 1355 | 5.7 |
| KBG pollen extract | 5 | 15380 ± 735 | 3.6 |
| | 10 | 26390 ± 792 | 6.1 |
| | 20 | 37780 ± 1867 | 8.7 |
| BSA | 5 | 6084 ± 1917 | 1.5 |
| | 10 | 9612 ± 1234 | 2.2 |
| | 20 | 16280 ± 2260 | 3.8 |
| β-galactosidase | 5 | 5590 ± 441 | 1.3 |
| | 10 | 8719 ± 460 | 2.0 |
| | 20 | 13880 ± 1177 | 3.2 |
| ConA | 5 | 38220 ± 3842 | 8.8 |
| | 10 | 68690 ± 5373 | 15.9 |
| | 20 | 3313 ± 444 | 0.77 |
| Culture medium | - | 4325 ± 1066 | 1.0 |

control, Con A, in contrast, demonstrated a different proliferation response of PLN cells, in view of the fact that the highest proliferation was observed at 10 $\mu$g/ml. However, RA induced PLN cells proliferation was not due to its association with $\beta$-galactosidase, which by itself induced significantly lower stimulation than RA. Similarly, the PLN cells from mice immunized by PBS with FCA or unrelated protein, such as BSA, showed little response to RA and KBG when cells were exposed to these Ags *in vitro* ( Fig. 13 ).

The RA and the natural KBG allergens ( KBG or KBG group 1 ) were compared for their abilities to specifically stimulate the murine PLN cells isolated from mice immunized with KBG. The results are summarized in Fig. 14. Clearly, the KBG at all concentrations (5, 10 and 20 $\mu$g/ml) exhibited the highest proliferation of PLN cells, as indicated by [$^3$H]-thymidine incorporation. Interestingly, the RA supported higher proliferation of PLN cells than KBG group 1 at all concentrations, suggesting that the RA may contain a dominant T cell epitope (s) of grass pollen allergens.

Figure 13. Proliferative response of PLN cells obtained from mice immunized with PBS or BSA

PLN cells were obtained from mice immunized with PBS or BSA and *in vitro* proliferation of PLN cells stimulated with different Ags at a dose of 5$\mu$g/ml. The [$^3$H]-thymidine incorporation was measured as counts per minute.

PROLIFERATION OF PLN CELLS OBTAINED FROM MICE IMMUNIZED WITH PBS OR BSA

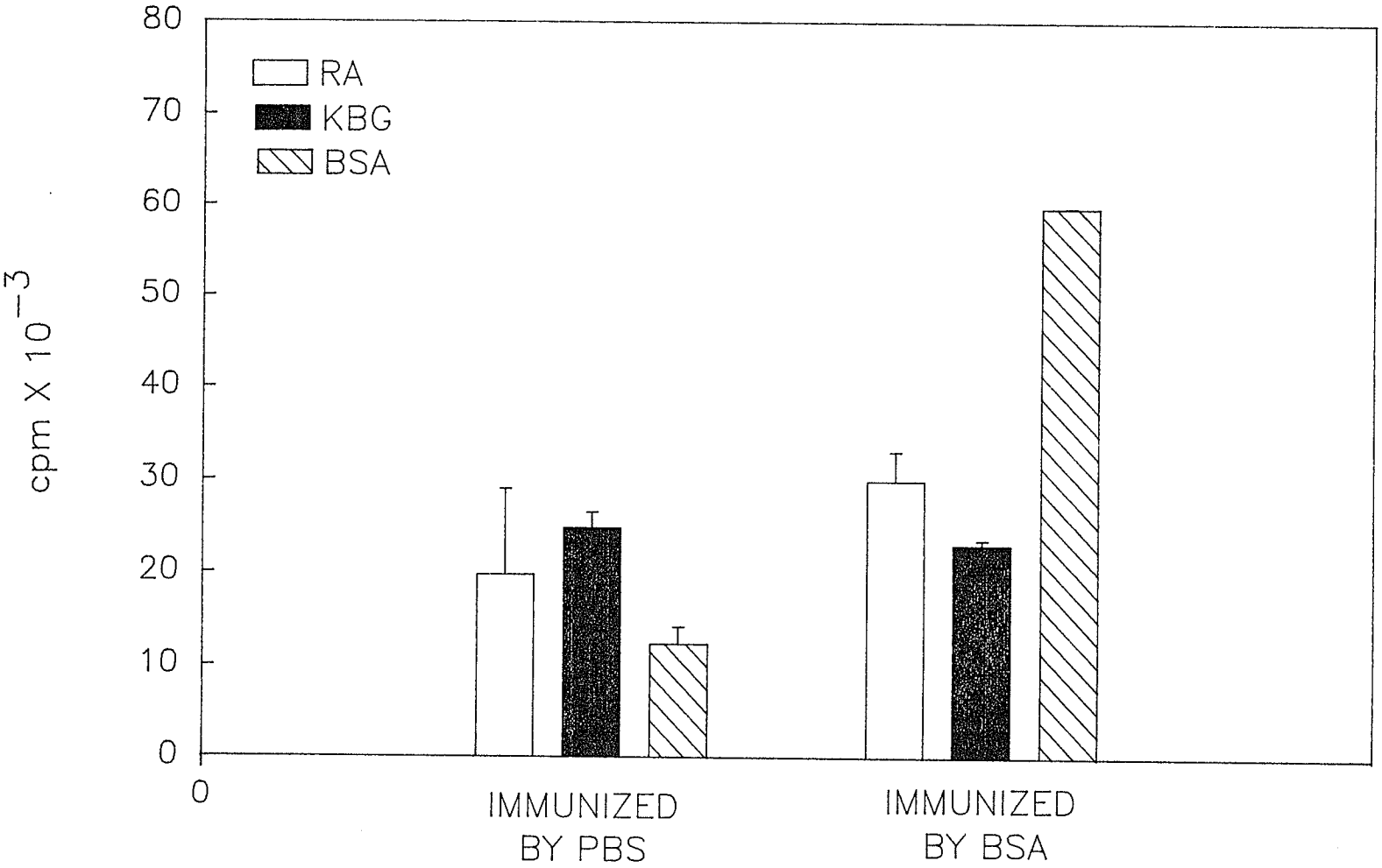



Fig. 13

Figure 14. Proliferation of PLN cells to RA, KBG and KBG group I

PLN cells were obtained from mice immunized with KBG ( 60$\mu$g/mouse ) and *in vitro* were stimulated with RA, KBG and KBG group I at different dosages. Proliferation was measured by [$^3$H]-thymidine incorporation.

PROLIFERATION OF PLN CELLS TO RA, KBG AND KBG GROUP 1

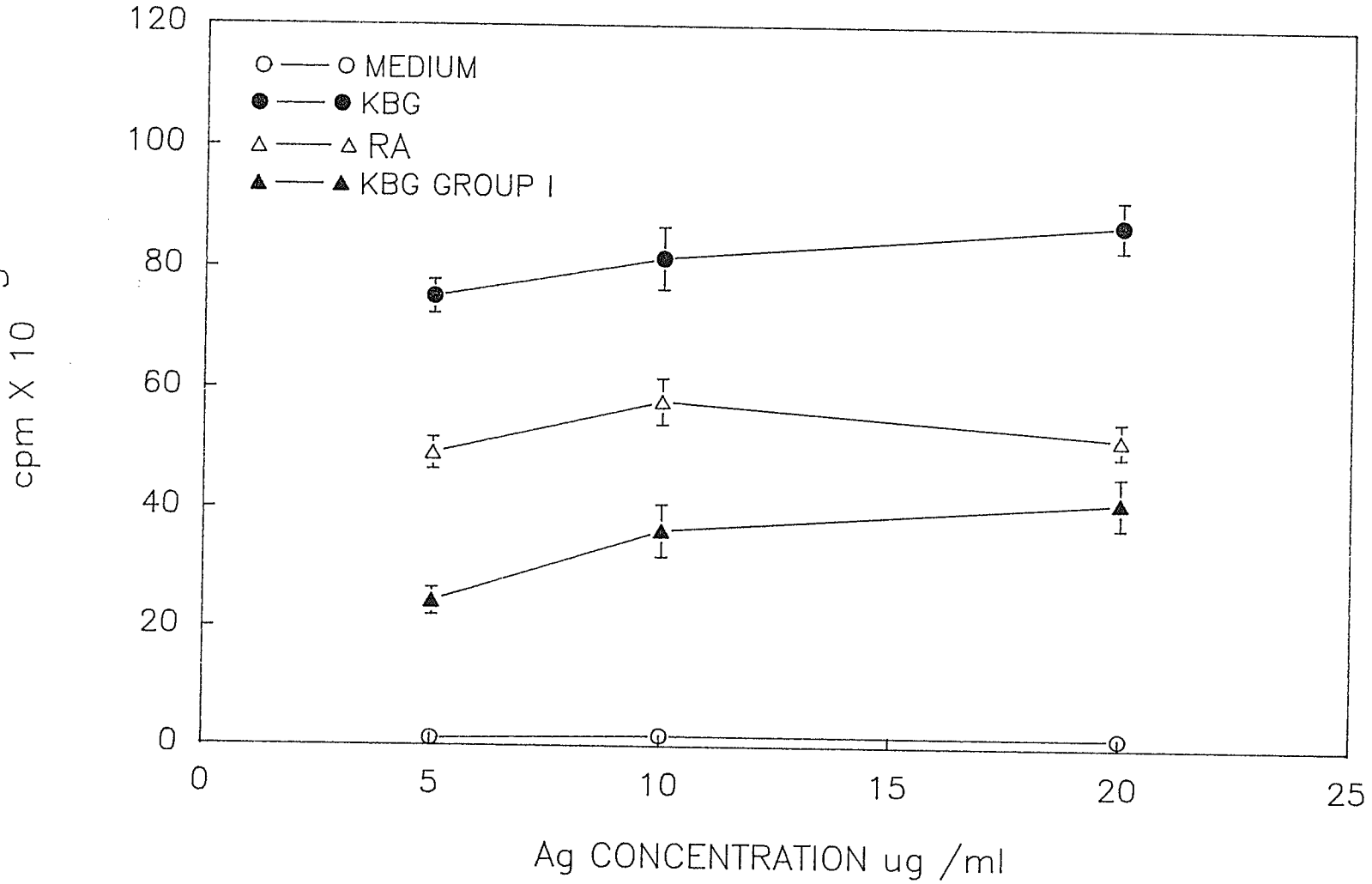


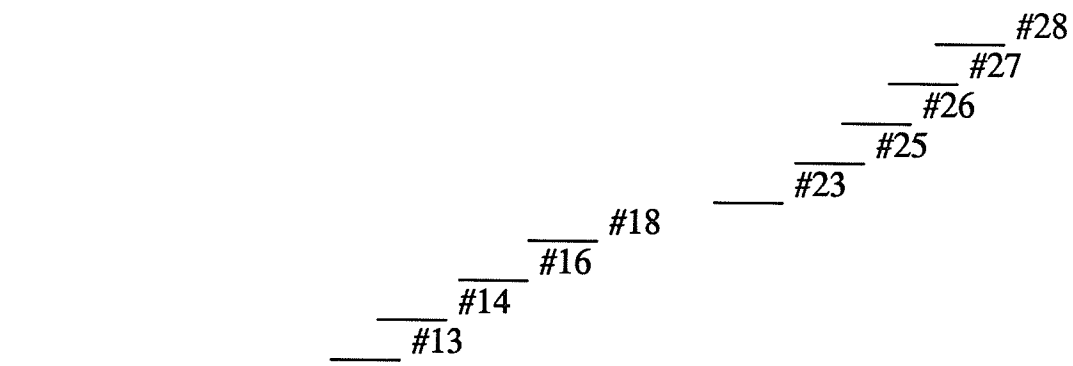

Fig. 14

(ii) Analysis of human T cell epitopes of RA

In murine system, it has already been established that RA could stimulate the proliferation of lymphnode cells . It is yet to be established that murine IgE has a similar role in mice to its role in hypersensitivity in humans.

According to the deduced amino acid sequence of the RA, overlapping peptides were produced by automated synthesis (kindly provided by Dr. Pele Chong, Connaught Lab. Limited). The peptides #13, #14, #16, #18, #23, #25, #26, #27 and #28 were represented the RA used in this study (Fig.15). The first strategy which we have used to identify T cell epitopes was to test the ability of synthesized peptides directly to stimulate PBMCs from grass pollen allergic patients. We investigated PBMCs responses to different concentrations of peptides #13 and #26. The maximum response obtained with each peptide was at 100 $\mu$g/ml (Fig. 16). This indicated PBMCs proliferated in a dose-dependent manner when stimulated with peptides. In total, eight patients' PBMC responses to nine peptides (as mentioned above) were tested. Results indicated that different individuals had varied levels of response to peptides. For each individual, the mean proliferative responses with at least three peptides showing the minimal dpm values was considered as backgroud. Except for three individuals, the rest of the group responded to peptide #13, while eight of nine subjects showed response to peptides either #25 or #26. Some individuals also responded to peptide #18, #23, #27, or

#28. The remaining peptides (#14 and #16) did not stimulate any significant responses (Fig. 17). This study allowed us to conclude that at least two regions on RA contained T cell epitopes (peptide #13 and #25, #26).




* __ 10 a.a.

Fig 15. Peptides encoding the sequences of the full length RA

According to the amino acid sequence of the RA, overlapping peptides were produced by automated synthesis. The full length RA comprised 303 amino acids and each peptide is 20 amino acids. The peptides #13, #14, #16, #18, #23, #25, #26, #27 and #28 encompassed the C-terminal conserved regions of the *Pao p* IX allergens.

Figure 16. Dose response curves to peptides #13 and #26

5 X 10 $^{5}$ PBMCs were cultured in the presence of different peptides at different amounts for five days. The values given are the arithmetic mean cpm of [$^{3}$H] thymidine incorporation in triplet cultures and the cpm to peptides are subtracted by background.

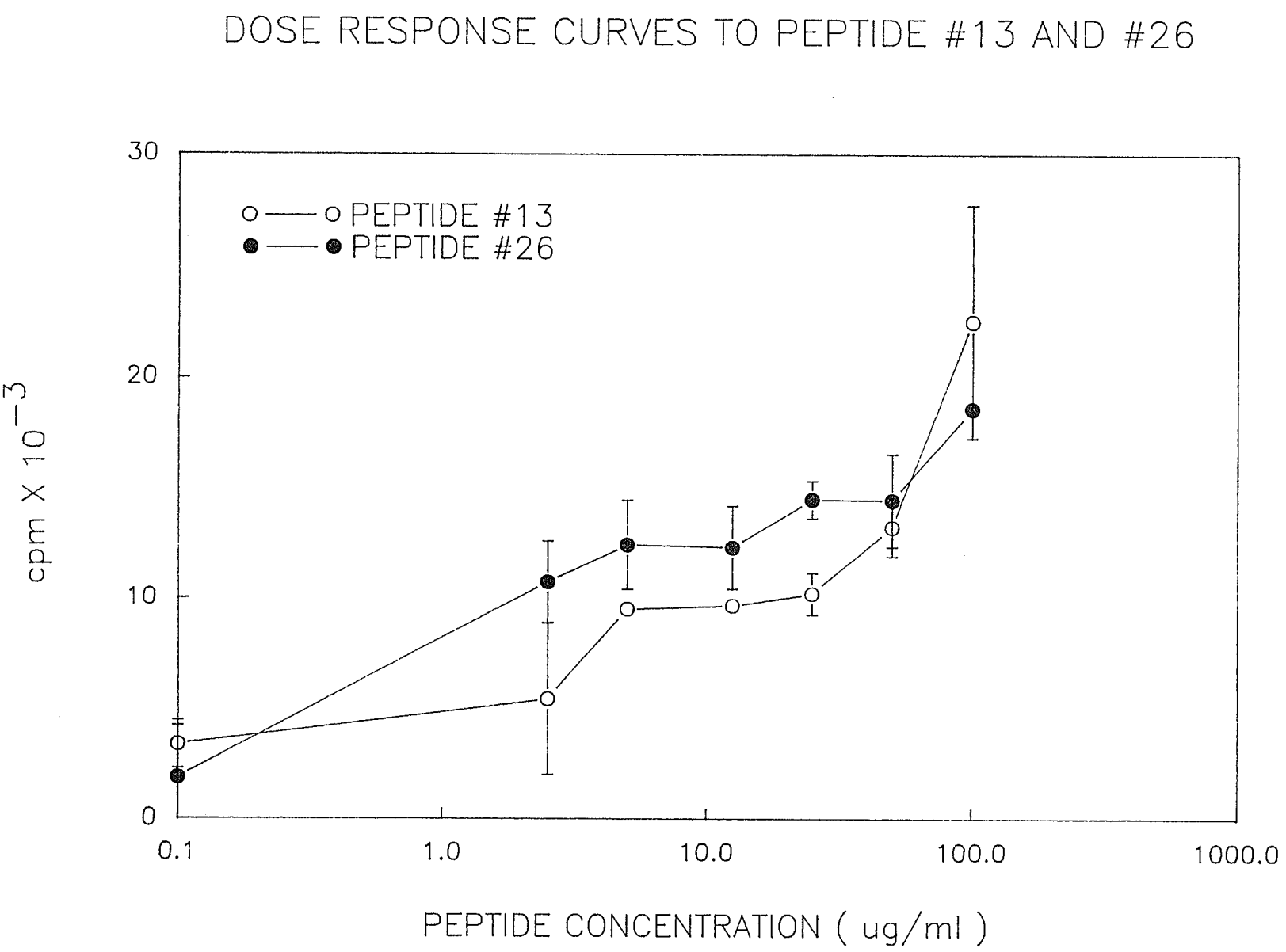

DOSE RESPONSE CURVES TO PEPTIDE #13 AND #26
PEPTIDE #13
PEPTIDE #26
cpm X 10$^{-3}$
30
20
10
0
0.1
1.0
10.0
100.0
1000.0
PEPTIDE CONCENTRATION ( ug/ml )

Fig. 16

Figure 17. PBMCs proliferation to peptides

5 X $10^5$ PBMCs were cultured in the presence of different peptides at 100 $\mu$g/ml for five days. The values given are the arithmetic mean cpm of [ $^3$H]-thymidine incorporation in triplet cultures and the cpm to peptides with background subtracted are shown.

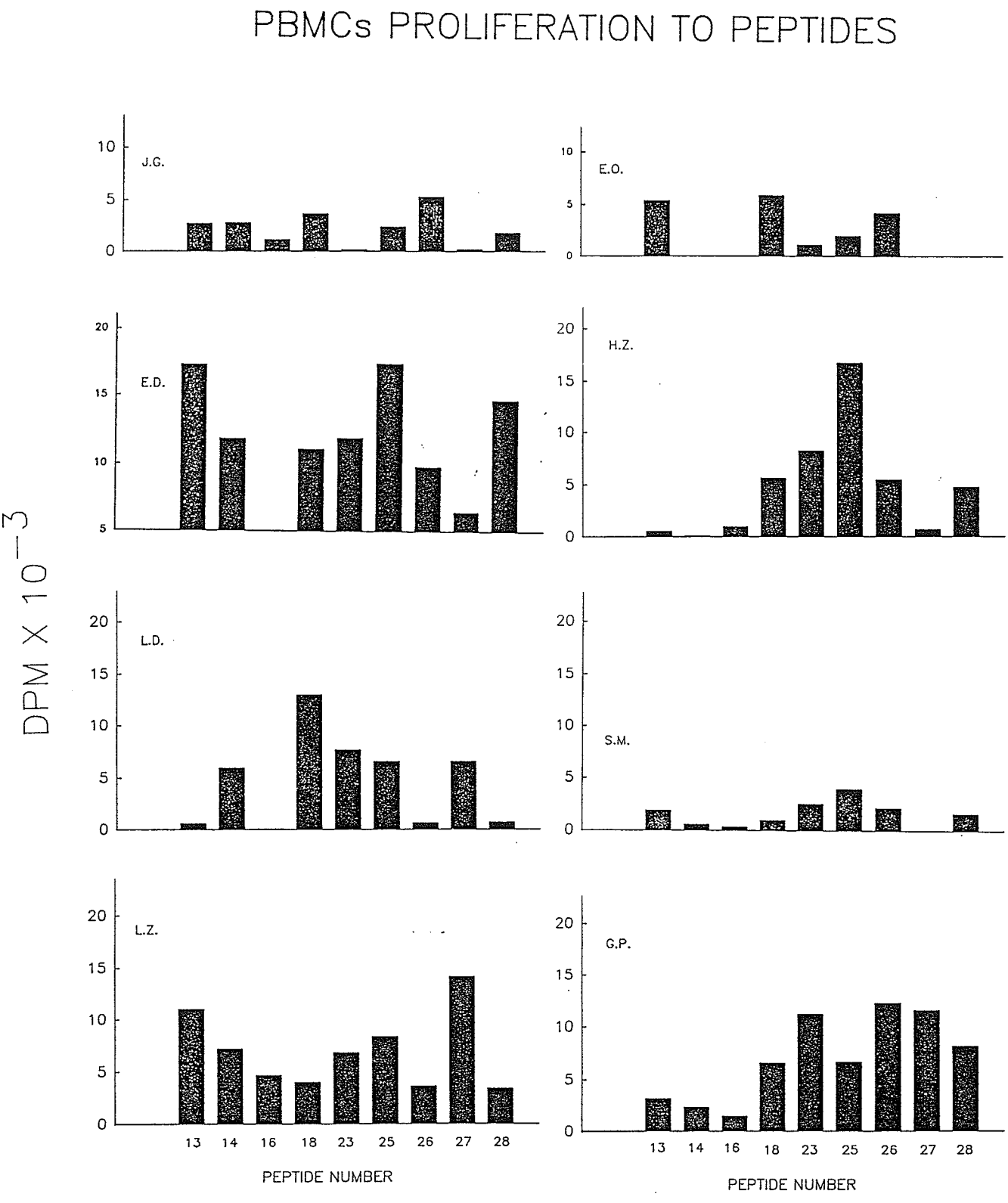

PBMCs PROLIFERATION TO PEPTIDES
DPM X 10⁻³
J.G.
E.O.
E.D.
H.Z.
L.D.
S.M.
L.Z.
G.P.
13 14 16 18 23 25 26 27 28
PEPTIDE NUMBER

Fig. 17

(iii) Studies with human T cell clones

(a) Optimization of protocols for T cell proliferation studies

Because proliferation of PBMC preparations from different individuals in response to peptide (s) is quite variable, antigen-specific T cell clone might overcome this disadvantage with strong responses to specific antigen and low background. But these T cell clones require repeated stimulation with Ag and accessory cells as well as with IL-2 (82). The need to obtain autologous PBMCs to restimulate such clones is time-consuming and demands ready access to the original T cell donor. This need may be obviated by using EBV-transformed B cell lines derived from the same T cell donor available for restimulation of the T cell as needed.

To examine the abilities and conditions of human EBV-transformed B cell lines to present allergens and induce allergen-specific T cell proliferation, first of all we used the Group I allergen specific T cell clones and autologous EBV-B cell lines (kindly provided by Dr. S. Romagnani, University of Florence, Italy). The results showed that autologous EBV-B cells were able to stimulate proliferation of the cloned T cells specifically to KBG and ryegrass antigens. But no proliferation was seen for T cell or B cell alone and T cell plus B cell (Fig. 18).

The antigen concentration for pre-incubation with EBV-B cells to induce the

Figure 18. EBV-transformed B cells are efficient APC for allergen

Two T cell clones, VI24 and VI33, were used in this assay. B cells (0.5 x $10^6$/ml) were pre-incubated at 37 °C, 5% $CO_2$ for 2-4 hours in the presence of allergens (KBG or ryegrass 50 $\mu$g/ml). Then, B cells were inactivated by mitomycin C and washed four times. T cells (1 x $10^6$/ml) and B cells preincubated with allergen were then co-cultured for 3 days. Proliferation was measured by [$^3$H]-thymidine incorporation ·

EBV–TRANSFORMED B CELLS ~~ARE~~ AS EFFICIENT APC ~~TO~~ FOR ALLERGENS

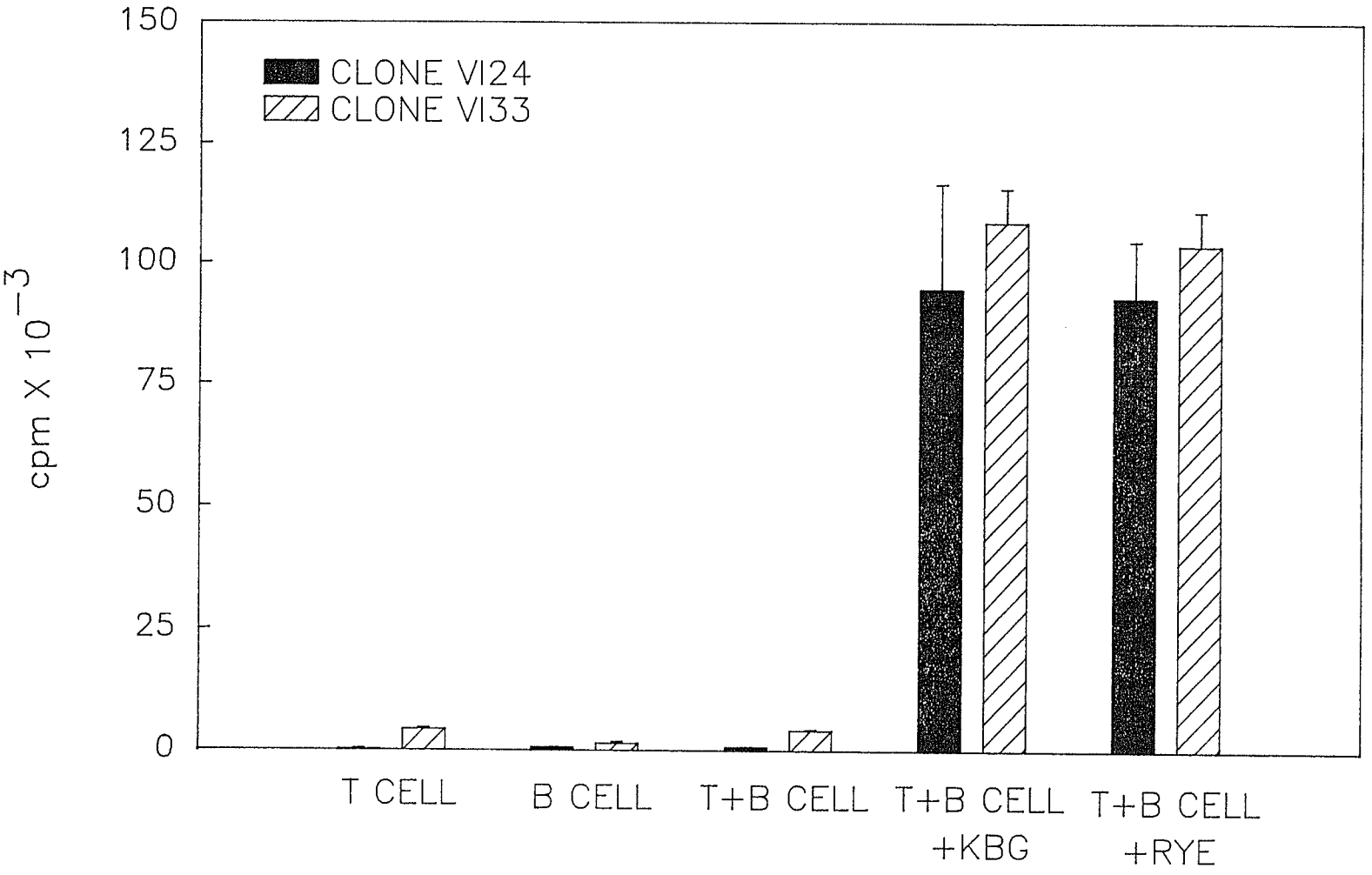



Fig. 18

proliferation of T cell clones was also optimised (Fig.19). Marked T cell proliferation was consistently observed to KBG or ryegrass antigens at the concentration 10 to 100 $\mu$g/ml, so in subsequent experiments 50$\mu$g/ml were used. There was a lower degree of proliferation with KBG or ryegrass at the antigen concentrations of 0.5, 1, and 5 $\mu$g/ml.

To investigate whether specific T/B cell cooperation, like Ag presentation by macrophage or nonspecific B cells (75, 76) is MHC-restricted, EBV-B cells were compared for their ability to present KBG to autologous or allogenic T cell clones. EBV-B cells were unable to present KBG to allogenic T cell clones (Fig. 20). This results suggested that the allergen-specific T / B cell interactions are MHC-restricted.

B cells were pre-incubated with allergen (KBG) for varying lengths of time, then inactivated by mitomycin C and cultured with T cells in the absence of allergen. B cells' ability to stimulate T cell clones measured by the incorporation of [$^3$H]-thymidine. B cell required approximately 120 minutes incubation with the allergen to maximally activate allergen-specific T cell clones. Longer than 120 minutes incubation of B cells with allergen did not increase T cell proliferation (Fig. 21). The rates measured presumably reflect the time required for processing initiated by pinocytotic uptake of allergen by B cells.

In Fig. 22, excess extracellular allergen (KBG or ryegrass) was added to the culture in which B cells had been pre-incubated with same allergen. T cell

Figure 19. Allergen concentration curves of B cell pre-incubation

EBV-transformed B cells were pre-incubated with a serial dilution of KBG or ryerass for 4 hours. Then, T cells and B cells were co-cultured for another 3 days. Proliferation was measured by [$^3$H]-thymidine incorporation .

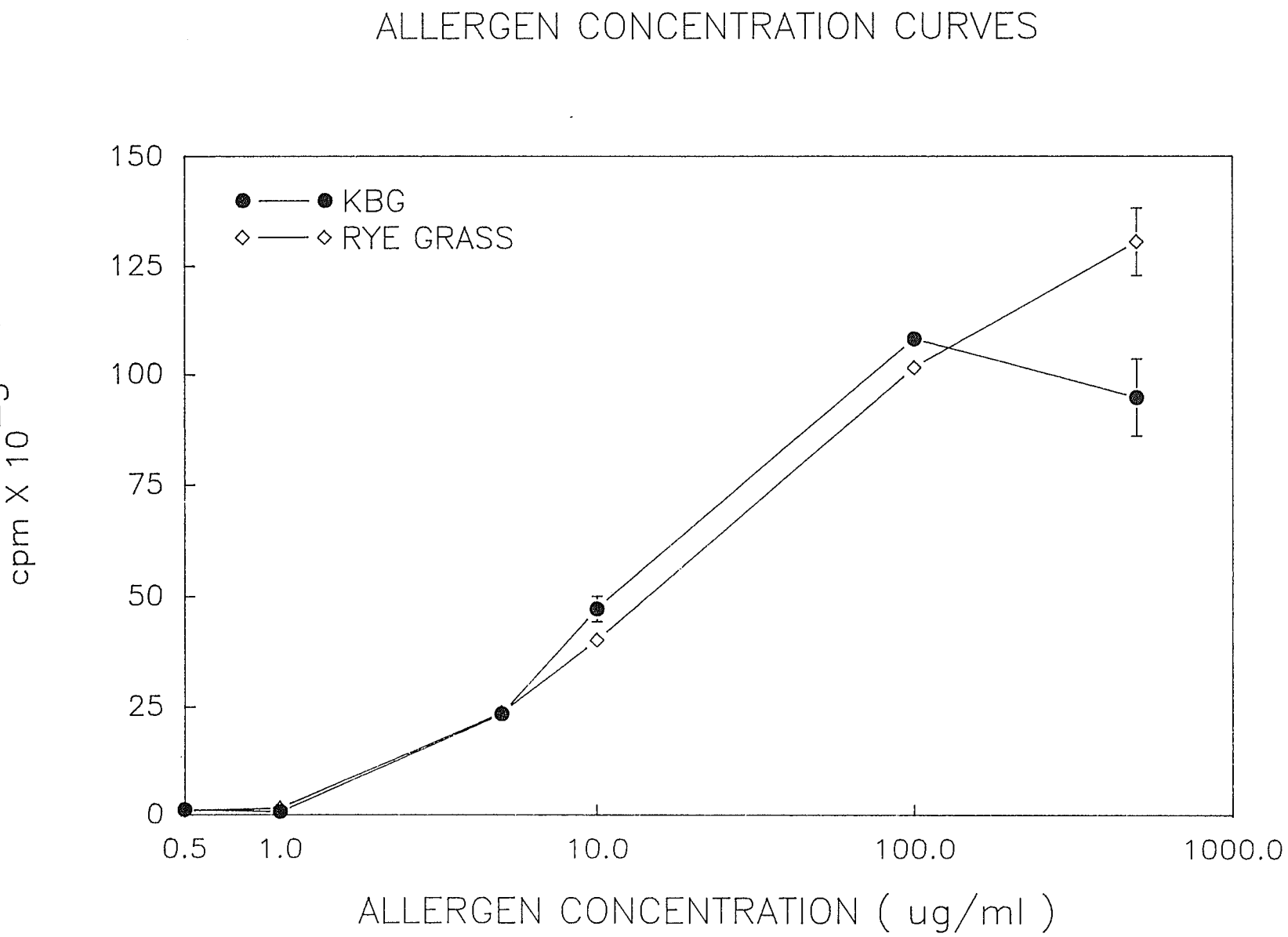

ALLERGEN CONCENTRATION CURVES
KBG
RYE GRASS
150
125
100
75
50
25
0
cpm X 10^-3
0.5
1.0
10.0
100.0
1000.0
ALLERGEN CONCENTRATION ( ug/ml )

Fig. 19

Figure 20. Analysis of T / B cell interaction

Autologous or allogenic EBV-transformed B cells were preincubated with KBG. Then auto- or allo- B cells were cultured with T cells. Proliferation was measured by [$^3$H]-thymidine incorporation.

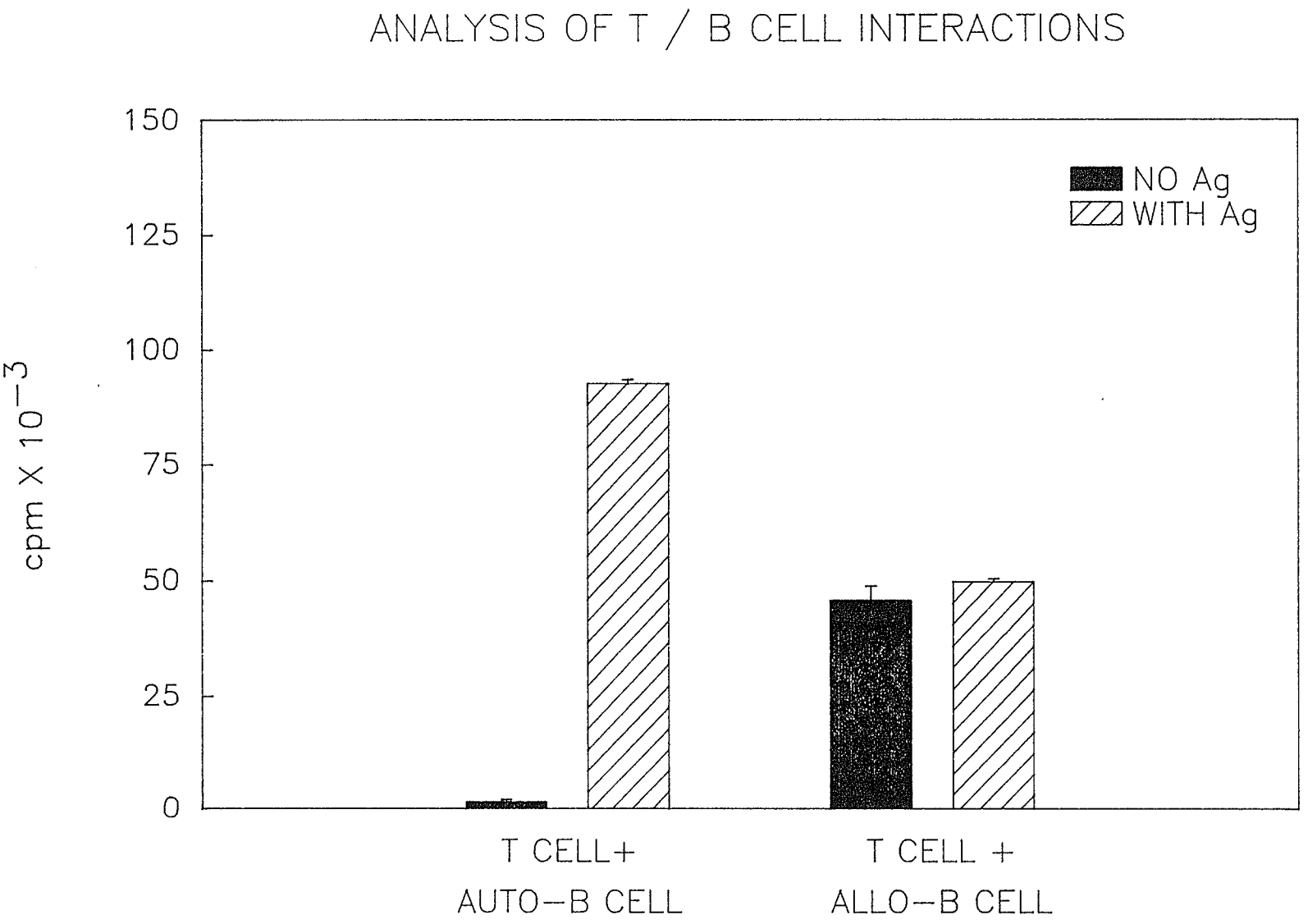

ANALYSIS OF T / B CELL INTERACTIONS
150
125
100
75
50
25
0
cpm X 10⁻³
NO Ag
WITH Ag
T CELL+
AUTO–B CELL
T CELL +
ALLO–B CELL

Fig. 20

Figure 21. Time course of pre-incubation allergen with B cells

EBV-transformed B cells (1 x $10^6$/ml) were incubated with KBG (50$\mu$g/ml) for different times (0 to 180 minutes) and then inactivated by mitomycin C. B cells and T cells were co-cultured for 3 days and cpm was measured after 16 hours of adding [$^3$H]-thymidine.

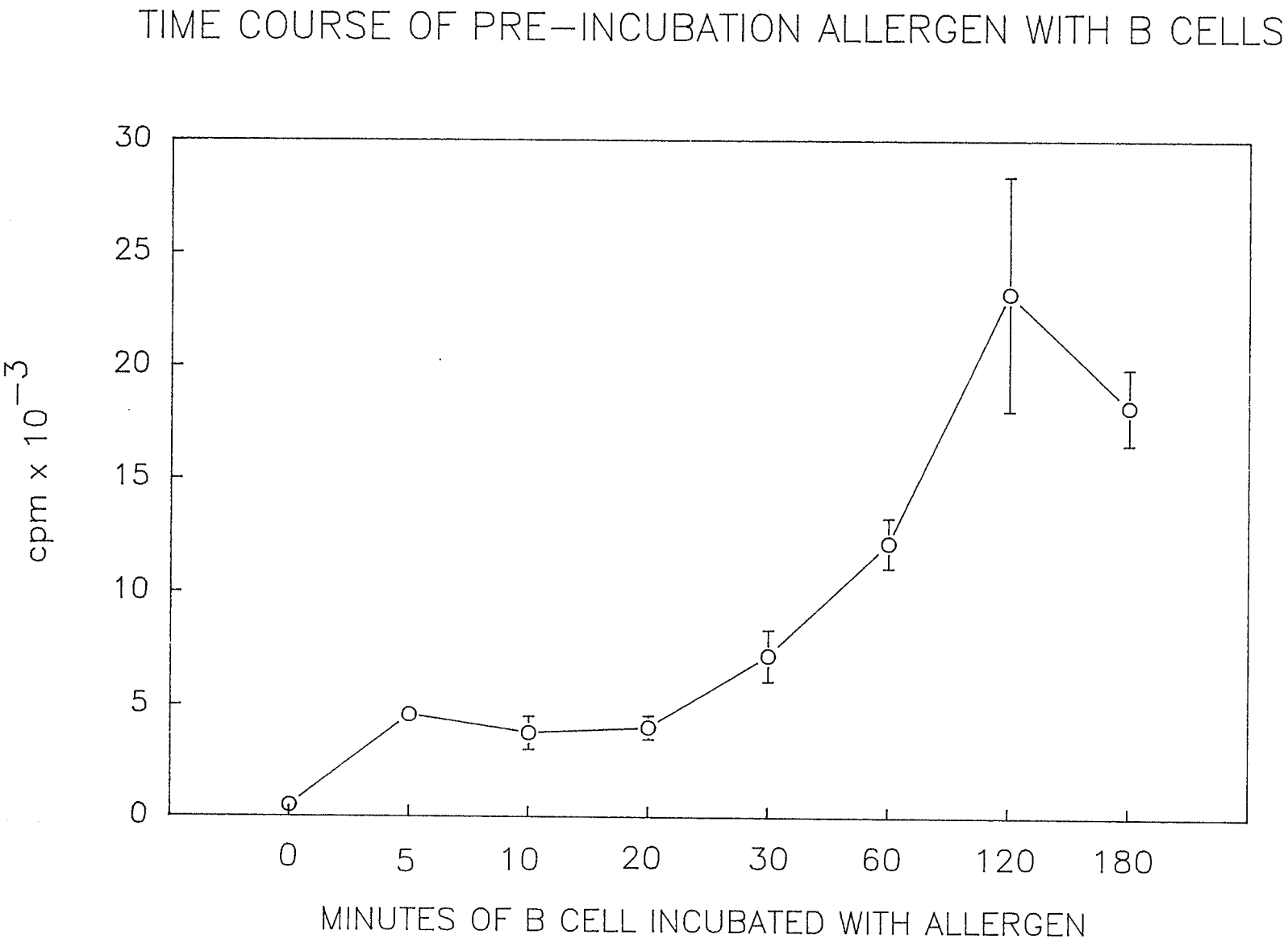

TIME COURSE OF PRE–INCUBATION ALLERGEN WITH B CELLS
cpm x 10^-3
30
25
20
15
10
5
0
0
5
10
20
30
60
120
180
MINUTES OF B CELL INCUBATED WITH ALLERGEN

Fig. 21

Figure 22: T cell clone responses to different allergen concentrations after B cells preincubated with allergen

T cells and B cells pre-incubated with KBG or ryegrass co-cultured in the presence of KBG or ryegrass at the different concentrations for 3 days. Proliferation was measured by [$^3$H]-thymidine incorporation.

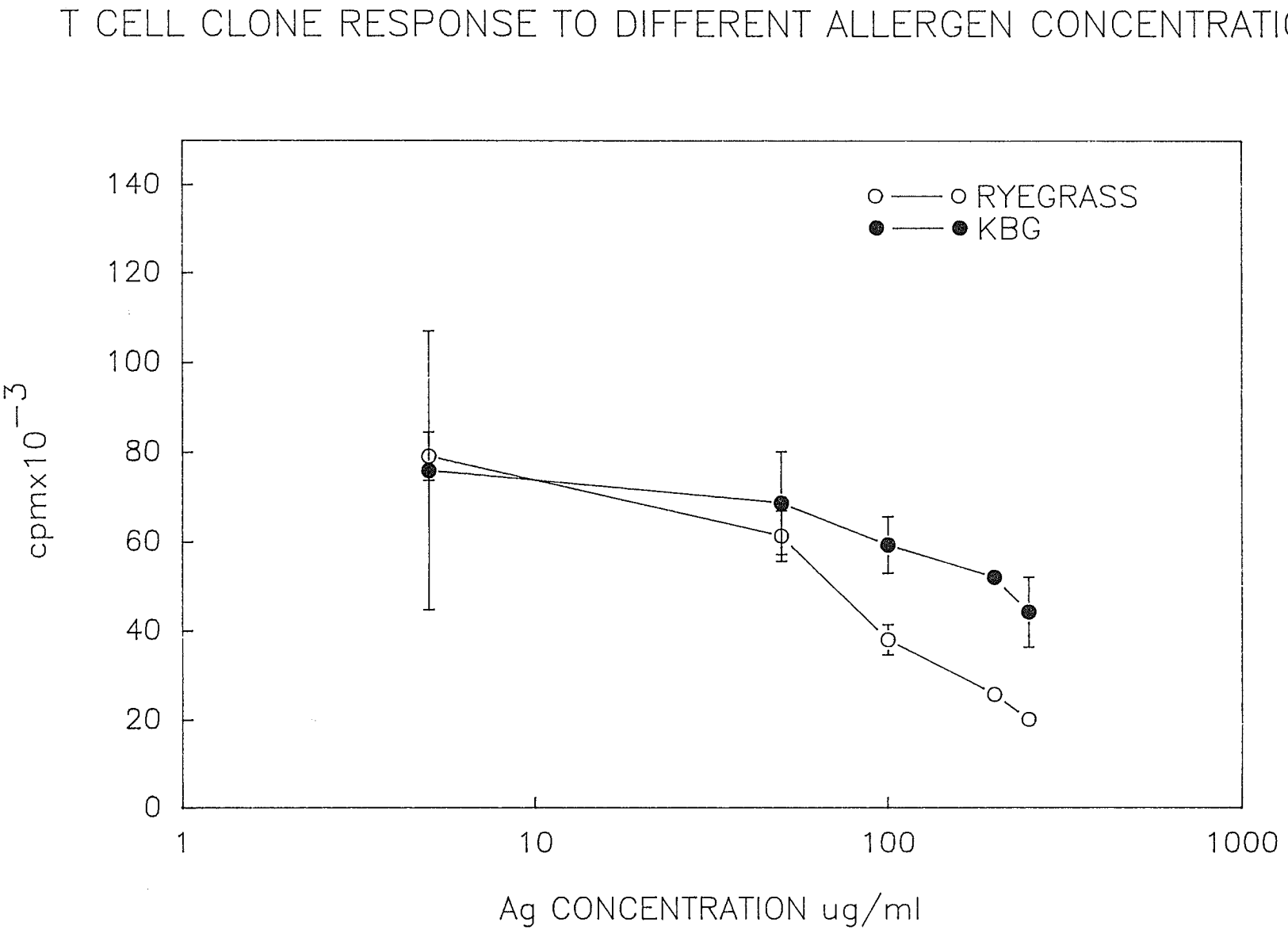

T CELL CLONE RESPONSE TO DIFFERENT ALLERGEN CONCENTRATIONS
RYEGRASS
KBG
140
120
100
80
60
40
20
0
cpmx10$^{-3}$
1
10
100
1000
Ag CONCENTRATION ug/ml

Fig. 22

proliferation did not show increase with increase in the concentration of allergen added to the culture, in contrast, T cell proliferation decreased as allergen concentration increased. This indicated that once the allergen has been processed in the context of MHC, exogenous allergen was not required during continued culture of B and T cells.

(b) Establishment and characterization of KBG-specific T cell lines and clones.

In our laboratory, KBG-specific T cell lines were established from allergic patient's PBMCs (Fig.23). Five of the clones we developed by limiting dilution from KBG-specific T cell-enriched lines appear to be specific to KBG allergen. Some additional clones appear to be autoreactive, that proliferation is identical in the presence or absence of allergen (Table. 5).

Three of the five allergen specific clones ( clone 6, 7, and 8 ) showed little response to KBG, if exogenous IL-2 was not added to the culture (Table. 6). However, after addition of exogenous IL-2 to the culture, these three clones showed significant increase in proliferation to KBG. These cloned T cells expressed IL-2 receptor and proliferated to antigen in the presence of exogenous rIL-2. That suggested those T cells might lack of endogenous IL-2 production.

Cell surface marker analysis of FACS demonstrated that 49% to 94% of total cell population of these five clones were CD3 positive. DR concentration varied from clone to clone and might be a marker for cell activation. DR positive cells

were found among the clonal cells from 25% to 83% of the total population. The major population of three clones were CD4 positive. The other two clones were found to be CD8 positive in the major cell population (Fig.24). These studies suggest that this protocol is capable of leading to the production of allergen specific clones.

Figure 23: KBG-specific T cell line proliferation to KBG

T cells from KBG-specific T cell line and EBV-B cells pre-incubated with KBG (50$\mu$g/ml) were cultured for 3 days and proliferation was measured by [$^3$H]-thymidine incorporation.

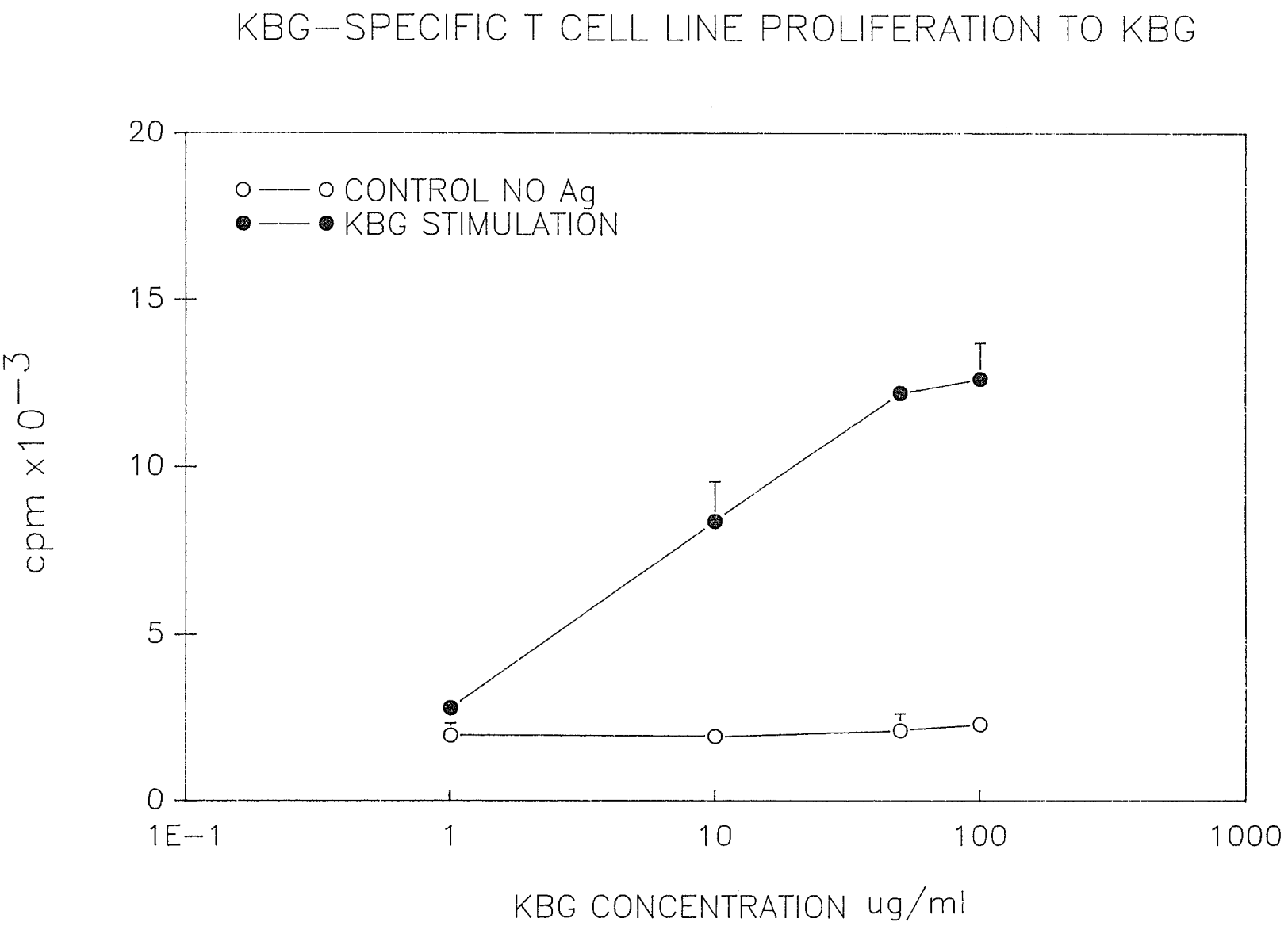

KBG–SPECIFIC T CELL LINE PROLIFERATION TO KBG
CONTROL NO Ag
KBG STIMULATION
cpm x10–3
20
15
10
5
0
1E–1
1
10
100
1000
KBG CONCENTRATION ug/ml

Fig. 23

Table: 5. Autoreactive clones

| | $^{3}$H-Thymidine incorporation cpm ± SD | |
|---|---|---|
| CLONE # | T + APC | T + APC + Ag |
| 1 | 7159 ± 106 | 7724 ± 270 |
| 2 | 5105 ± 603 | 6738 ± 580 |
| 3 | 8067 ± 329 | 9316 ± 222 |
| 4 | 6958 ± 599 | 7824 ± 181 |
| 5 | 8702 ± 1612 | 8767 ± 1001 |

Table: 6. Ag-specific clones

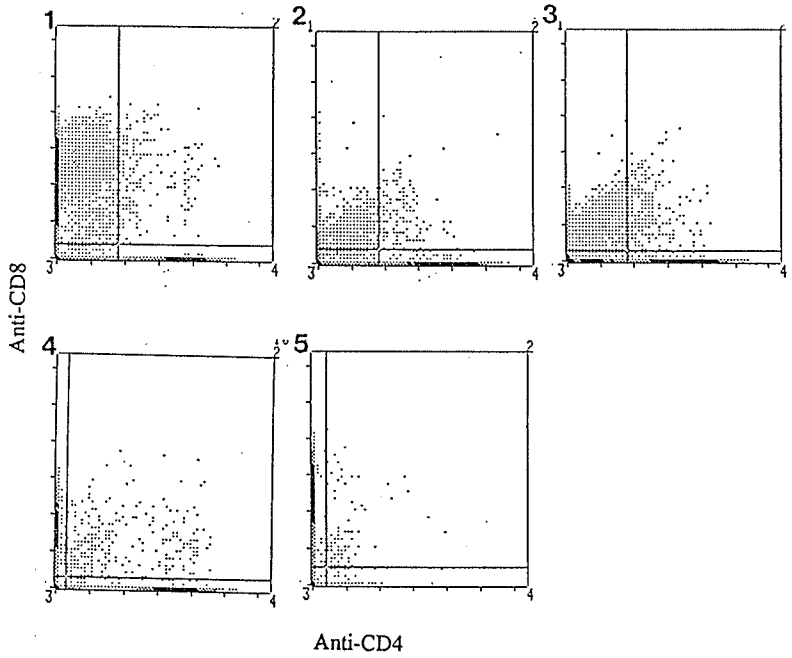
| | [$^3$H]-thymidine incorporation cpm ± SD * | |
|---|---|---|
| CLONE # | T+APC+Ag | T+APC+Ag+IL-2 |
| 6 | 1730 ± 677 | 4041 ± 2683 |
| 7 | 1623 ± 74 | 7641 ± 3174 |
| 8 | 877 ± 104 | 36,174 ± 5875 |
| 9 | 17,394 ± 2563 | 16,345 ± 116 |
| 10 | 10,730 ± 2004 | 15,357 ± 1749 |

* All values were subtracted by background:

The cpm of T cell + B cell + Ag was subtracted by the cpm of T cell + B cell. The cpm of T cell + B cell + IL-2 + Ag was subtracted by the cpm of T cell + B cell + IL-2.

Figure 24. T cell markers expressed on five allergen-specific T cell clones

T cells were examined for CD3, CD4, CD8, and DR expression by two colour flow microfluorometric analysis. The flow cytometer on cells was gated with the scatter characteristics of lymphocytes. 10,000 cells were counted and the results were expressed on the basis of two-colour histograms as % CD4-FITC positive cells (green; X) vs % CD8-Rhodamine positive cells (red; Y) and % CD3-FITC positive cells (green; X) vs % DR-Rhodamine positive cells (red; Y) within the cell population.




Clone #1, CD4-18.31%, CD8-61.64%;

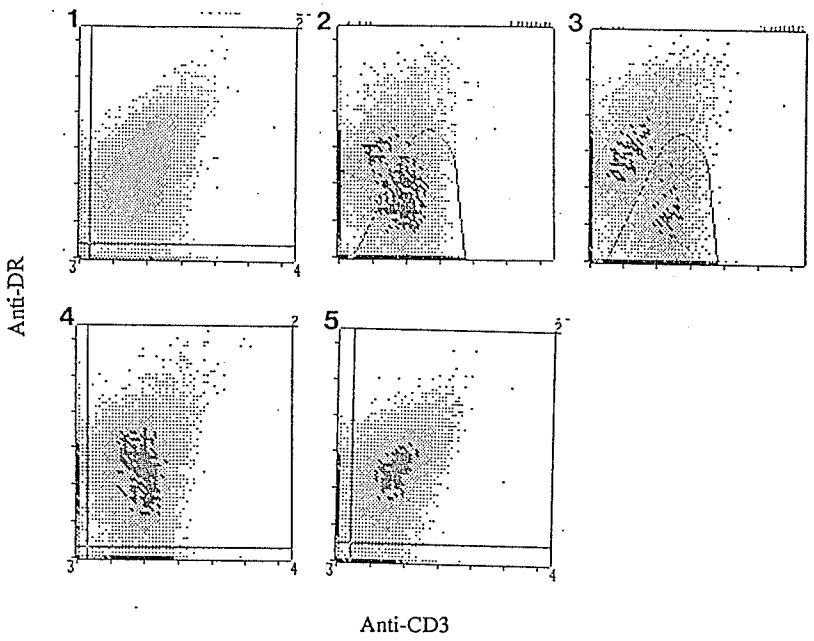
Clone #2, CD4-69.17%, CD8-5.86%;

Clone #3, CD4-49.81%, CD8-14.55%;

Clone #4, CD4-45.53%, CD8-29.18%;

Clone #5, CD4-70.9%, CD8-0.65%.

Fig. 24 ( a )




Clone #1, DR-73.31%, CD3-83.91%;

Clone #2, DR-36.48%, CD3-59.1%;

Clone #3, DR-25.91%, CD3-49.11%;

Clone #4, DR-69.21%, CD3-80.49%;

Clone #5, DR-67.37%, CD3-75.76%.

Fig. 24 ( b )

# V. DISCUSSION

Recently, the use of recombinant DNA technology has greatly stimulated the study of allergens and has become the most important tool for allergen characterization. Both diagnosis and therapy of allergic diseases caused by specific allergens are expected to benefit substantially from the use of cloned allergens : (i) pure recombinant allergens can be produced in virtually unlimited quantities (ii) DNA sequences of cDNA-clones can be determined much more easily than the corresponding protein sequences and provide the basis for epitope mapping and (iii) synthetic peptides corresponding to mapped epitopes possess significant potential for *in vitro* and *in vivo* diagnosis, immunotherapy and possible new forms of treatment (83). Moreover, cDNA-clones and sequences of allergen are a prerequisite for investigating the biological functions and the regulation of gene expression of allergens. The definition of structural determinants of proteins capable of eliciting IgE-responses would make it possible to investigate the mechanisms underlying IgE Absynthesis.

In view of the above, a number of investigators have recently undertaken studies to clone allergenic proteins and synthesize the corresponding RA. However, detailed studies of the molecular and structural properties of these

allergens is limited. The main purpose of this study was to characterize the recently cloned *Poa p* IX proteins, which constitute a major group of allergens of grass pollen. The RA encoded by cDNA clone KBG7.2 which comprised the conserved region of all *Poa p* IX allergens was used for this purpose. This study focused on the immunologic characterization of the RA, in respect of (i) its allergenic and antigenic properties, and (ii) T cell responses to this Ag and several overlapping synthetic peptides derived from this Ag.

It is noteworthy that in a parallel, but complementary study the recombinant allergenic peptide has enabled us to identify the pollen proteins encoded by the KBG7.2-homologous cDNAs. Although these proteins appeared to be present in barely detectable amounts in this batch of pollen, as is evident from the SDS-PAGE electrophoretogram, the production of this recombinant peptide in large amounts made possible the identification of the natural allergenic proteins in KBG pollen (84). The fact that about three polypeptides were recognized by the IgE Abs corresponding to the RA indicated the existence of a group of isoallergenic proteins in KBG pollen. It is noteworthy that further characterization clearly established this group of proteins as a new group of allergenic proteins of grasses, which on the basis of existing framework International Allergen Numerelature System have been designated as *Poa patensis* IX (*Poa P* IX) group of proteins. In the FP expressed in *E. coli*, the RA is contaminated by *E. coli* proteins. Therefore, purification of the RA was necessary for further study. The method we

used was to excise the 81 kDa FP band from SDS-PAGE and elute the protein using a special buffer. Results showed this method proved to be simple and fast.

A major finding from this study is the positive correlations in IgE antibody reactivities of RA and KBG proteins and of RA and mixture of ten grass pollen extracts. Specific IgE level to KBG as well as RA of fifty-five patients' sera corresponded to clinic SPT results; a higher SPT score was associated with higher specific IgE binding. The observed positive correlation for IgE reactivity of the cloned peptide and total crude extract suggests that the cloned allergenic peptide is a major component of grass pollen. It is apparent from the correlation studies of IgE reactivity with RA versus KBG and RA versus grass pollen mixture that the highly purified recombinant allergens not only may facilitate an accurate diagnosis of allergy by *in vitro* tests (RIA or ELISA), as is presented in here, but also may aid discriminatory diagnosis of allergy to different grass-pollen components by SPTs.

The positive correlation between grass mix and RA with respect to IgE binding raised the possibility of cross-reactivities among pollen allergens. Indeed, in a separate study, it was shown that the *Poa p* IX allergens existed in 8 out of 13 grass extracts examined (85). Moreover, the relative binding and inhibition analyses revealed that the group IX allergens are similar with respect to their antigenic and allergenic epitopes (85).

Isotype-specific reactivities of these allergenic proteins was further examined

by ELISA. Thus KBG and RA specific IgE (n=978), IgG4 (n=978) and total IgG Abs (n=350) were measured by application of anti-IgE, anti-IgG4 subclass and anti-IgG antibodies were investigated. The aim of this study was to determine whether IgE antibody formation parallels IgG4 or IgG antibody formation in allergic patients.

There are a number of functional differences between the different human IgG subclasses. while IgG1 and IgG3 antibodies fix complement, IgG2 and IgG4 do not. The function of these antibodies that do not fix complement is unclear (86). Previous studies have shown that repeated exposure antigens or repeated antigenic stimulation during allergen immunotherapy often results in an IgG4-restricted response (87-88). IL-4 has been shown to have pleiotropic effects on B and T lymphocytes as well as on other cell types (89,90), in humans, in addition to the enhancement of IgE synthesis, IL-4 preferentially induced IgG4 synthesis *in vitro* (91). The concomitant induction of IgE and IgG4 suggests that the regulation of these two isotypes may be linked. The mechanisms through which IL-4 enhances IgG4 synthesis still remain unclear. In the murine system, it has been suggested that IL-4 affects IgE and IgG1 production at the level of isotype switching but not in the regulation of cell growth (92).

The sera of 978 allergic patients were also examined for their reactivities with human IgE and IgG4 Abs. KBG specific IgE and IgG4 showed a positive correlation (r=0.53). The same was found for RA specific IgE and IgG4 Abs,

although the r value (=0.35) was lower than that for KBG specific Abs. This result demonstrated that IgE antibody formation parallels IgG4 antibody formation in atopic humans *in vivo (30)*. It has been reported recently that the proteins which bind to IgE antibodies also bind to IgG4 antibodies (93). Our results are in agreement with this report. Also our finding corresponds well with other investigators' finding that IL-4 enhances IgE and IgG4 synthesis *in vitro*.

Positive correlations were demonstrated between KBG and RA with respect to both IgE and IgG4 antibody reactivities. This suggests that RA contained not only IgE, but also IgG4 binding sites of natural KBG proteins. Such findings have important clinical significance. In this case RA can be used in the evaluation of the outcome of the treatment involving allergen specific immunotherapy. For this purpose, RA is better than natural KBG because of its unlimited quantities and the specificity. The reason of this is that it is reported that a patient's predominant IgG4 response after immunotherapy resulted in a poor amelioration of clinical symptoms, which implies that they probably formed both IgG and IgG4 antibodies to the allergen injection (94).

Persuant to the studies of antibody reactivities, the cellular responses to the RA was investigated. Initial studies focused on proliferation of murine lymphocytes, since it was demonstrated that RA readily induced IgE antibodies in mice. Thus proliferation responses elicited by KBG and RA were examined using murine PLN cells. The C3H / HeJ strain was chosen for analyzing the cellular response to KBG

and RA because previous studies (95, 96) demonstrated that a antibody response to grass pollen allergens could be readily obtained in this strain of mice. Our data have clearly indicated that a T-cell proliferative response was readily elicited in antigen-primed cells by KBG and RA. The specificity of KBG and RA was confirmed by little or no response in the presence of unrelated proteins *in vitro*. Conversely, KBG or its peptides did not stimulate any proliferation of cells from mice that had been primed with PBS as control or an unrelated protein, such as BSA, even with Freund's complete adjuvant. The proliferation of murine PLN cells in response to RA suggests the existence of T cell recognition structures on the recombinant peptide. However, this study also demonstrated that the RA retained the immunogenicity of the original KBG pollen in terms of its ability (i) to induce the *in vivo* priming of PLN cells and (ii) to elicit the *in vitro* proliferation of the antigen-primed PLN cells.

It is yet to be established that murine IgE has a similar role in mice to its role in hypersensitivity in humans. However, it was found that both human and mouse IgG was recognized similar epitopes on allergenic molecule (97) and that some of these epitopes also recognized murine T cells. The results of antibody production (81) combined with the above results have clearly led to the establishment of a murine model, this model may be useful for examining the efficacy of therapy involving recombinant allergens with or without their chemical modification and for studies aimed modulation of IgE antibodies by novel approaches.

The investigation of T cell epitopes that enhance the switch to IgE antibody production is critical to our understanding of allergenic properties of RA. Recognition of antigen by T cells requires processing and presentation of short linear peptides. As peptides must also be recognized by T cell receptor, the prediction of T cell epitopes based only on their ability to bind to MHC molecules is insufficient and at present more direct approaches must still be used for the localization of T cell epitopes in allergens. Historically, empirical epitope mapping of protein antigens relied upon either enzymic digestion or cyanogen bromide cleavage into successively smaller fragments retaining epitopic specificity. Later, advances in peptide synthesis technology, and the advent of molecular biology techniques brought epitope mapping more sharply into focus. We used of synthetic peptides to screen the T cell repertoire reactive with allergen. The PBMC from majority of the grass pollen allergic subjects were tested with a number of peptides. Interestingly, the pattern of stimulation varied from subject to subject, however, a majority of them responded to peptides #13 and #25, 26. This revealed that at least there two regions on RA are capable of stimulating T cells in a number of subjects. These may represent immunodominant epitopes.

The variability of human PBMC responses to peptides encouraged us to establish allergen-specific human T cell clones. But allergen specific T cell clones require repeated restimulation with antigen and accessory cells. The need to obtain autologous PBMC to restimulate such clones (every 7-10 days) is time-consuming

and not convenient. Our data showed the use of EBV-B cell immobilized line as antigen-presenting cells for grass pollen should be especially valuable in studies requiring the continuous propagation of allergen-specific human T cell clones *in vitro*.

It appears that all cells which normally express class II molecules, such as B cells, macrophages and dendritic cells, could function as antigen presenting cells. However, B cells have many of the qualifications for APC: (i) they carry surface antibodies that permits them to selectively bind antigen; (ii) they show constitutive expression of class II MHC molecules; (iii) they internalize proteins and degrade them; and (iv) they produce at least one costimulator, IL-1 (98). In order to obtain immobilized B cells, EB virus was used to transform B cells into continuously proliferating B lymphoblastoid cell lines. Our EBV-B cell lines showed 99% CD40 (a human B cell marker) positive by FACS. The data proved pure B cell lines have ability to stimulate autologous allergen specific T cell proliferative responses. EBV-B cell lines then presented to T cells in an MHC-restricted manner indistinguishable from that characteristic of other conventional APCs.

The present study raised a number of questions. B cells bind the antigen either via surface immunoglobulin or non-specifically binding. It seems that EBV-B cells, which function as APC, may bind Ag non-specifically, because our EBV-B cell lines failed to selectively bind radiolabeled KBG or RA and to secrete any antibody after 2 months culture *in vitro* when they were used as antigen presenting cells

(data not shown). In this case, the concentration of antigen required to bind B cells are several-fold higher than in the specific binding through surface immunoglobulin (99-101). it is now well established that B cells are efficient APC *in vitro*. EBV-B cell lines have been shown to present grass pollen to T cells. EBV-B cell lines functioned as APC like macrophages through non-specific binding antigen. Macrophages and non-specific B cells must usually first internalize and process a complex protein antigen before presenting it to T cells.

We have demonstrated that T cells from grass pollen-allergenic individuals can respond to the KBG allergens in a specific manner. Long-term allergen-specific genetically restricted, T cell lines can be readily established from the peripheral blood of grass pollen responsive individuals. These allergen-specific lines can be cloned out by limiting dilution. Some of these clones are CD4 positive, but others are CD8 positive.

A cloned T cell system is required to study these allergen-specific responses. This system potentially allows, at the clonal level, identification of the nature of the antigenic determinants (T cell epitopes) recognized by the allergen-specific T cells. However, autologous EBV-B cells alone induced significant T cell proliferation in the absence of added antigen. Because very similar results were observed in most of the clones, it was concluded that some T cells may be recognizing antigen on the surface of the autologous EBV-B cells. These antigens could be related to EBV itself or could be neoantigens expressed on B

lymphoblasts. Our studies suggest that clones can be readily produced using EBV transformed B cells. However, this procedure leads to the production of autoreactive clones.

# VI. CONCLUSIONS

The humoral and cellular immunologic methods employed in this study for characterization of RA encoded by cDNA KBG7.2 have demonstrated that this RA constitutes a major allergenic protein of grass pollens and may be of diagnostic and therapeutic value.

On western immunoblot, the IgE antibodies in a pool of sera from allergic patients reacted strongly with the 81 kDa band present in RA producing *E. coli* extract. The constituent RA of this FP corresponded to KBG pollen proteins with M.W. 28 to 34 kDa which have been designated as *Poa p* IX. The method of purifying FP from *E. coli* extract by cutting and eluting protein from SDS-PAGE which we used in this study proved to be simple and fast.

The observed positive correlation for IgE reactivity between the RA and KBG pollen extract or mixed crude pollen extracts of ten grasses and high percent inhibition of IgE binding ability of natural KBG by RA suggest that the recombinant allergen is a major component of grass pollens and can be employed for diagnosis of grass pollen allergy.

The high scores in skin prick test were associated with high specific IgE levels, irrespective of the antigens (KBG pollen extract or RA) indicated the highly purified recombinant allergen could facilitate an accurate diagnosis of allergen *in*

*vitro* and *in vivo*.

Associations between IgE and IgG4 Ab reactivities of KBG and RA in a population of 978 allergic subjects indicated that IgE antibody formation parallels IgG4 antibody synthesis in atopic humans *in vivo*. This finding may be used to evaluate the outcome of the treatment involving allergen-specific immunotherapy.

Studies with murine PLN cells showed that RA and KBG equally stimulated the proliferation of these cells indicating that RA possessed structures capable of recognizing T cells. The human PBMC proliferation studies using synthetic peptides demonstrated that T cell responses of each individual differed, however, most of the individuals responded to peptides #13 and #25. These results revealed that at least two regions on RA has T cell stimulatory activity which may comprise immunodominant epitopes.

EBV-B cells can process and present allergen to T cells and induce T cell proliferation. Allergen specific human T cell lines or clones can be established by using EBV-B cell lines as antigen presenting cells.

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