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CHARACTERIZATION OF GROUP I ALLERGENS OF KENTUCKY BLUE GRASS AND RYE GRASS POLLENS WITH MONOCLONAL ANTIBODIES

BY

© ZHENGWEI LIN

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BY

ZHENGWEI LIN

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

MASTER OF SCIENCE

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LIST OF ABBREVIATIONS:

AB:	assay buffer
Ag:	antigen
APC:	antigen presenting cell
BSA:	bovine serum albumin
CNBr:	cyanogen bromide
cpm:	counts per minute
E:	epitope
EIA:	enzyme immunoassay
ELISA:	enzyme-linked immunosorbent assay
FPLC:	fast protein liquid chromatography
hr:	hour
HLA:	human leukocyte antigen
HRP:	horseradish peroxidase
IB:	incubation buffer
IEF:	isoelectrofocussing
Ig:	immunoglobulin
i.p.:	intraperitoneally
KBG-R:	retentate fraction of Kentucky Blue grass pollen
Lol p:	*Lolium perenne* (Rye grass pollen)
Mab:	monoclonal antibody
min:	minute
M.W. or Mr:	molecular weight or relative molecular weight
OD:	optical density

LIST OF ABBREVIATIONS CONTINUED:

PBS:	phosphate buffered saline
PGD_2:	prostaglandin D_2
pI:	isoelectric point
Poa p:	*Poa pratensis* (Kentucky Blue grass pollen)
RAST:	radioallergosorbent test
RIA:	radioimmunoassay
SDS-PAGE:	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SRS-A:	slow-reacting substance of anaphylaxis
TBS:	Tris buffered saline

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PREFACE

The main objective of the present study is to isolate and characterize group I type antigen from Kentucky Blue grass pollen, named *Poa p* I and to compare the antigenic and allergenic relationships of *Poa p* I and *Lol p* I (group I type antigen from Rye grass pollen) antigens using monoclonal anti-*Poa p* I and anti-*Lol p* I antibodies as well as allergic human sera.

This thesis is presented in three chapters. Studies presented in Chapter I describe the isolation of *Poa p* I antigen from Kentucky Blue grass pollen by using a monoclonal anti-*Lol p* I antibody, Mab 290A-167. *Poa p* I was preferentially characterized with respect to its antigenicity and allergenicity as well as its physicochemical properties. Furthermore, the preparation of monoclonal antibodies specific for the purified *Poa p* I is also reported in this chapter.

Chapter II describes the relative relationships of the antigenic and allergenic epitopes on *Poa p* I antigen which are determined by cross-inhibition assay with monoclonal antibodies and human IgE antibodies. The homologies of antigenic and allergenic specificities between *Poa p* I and *Lol p* I have also been investigated.

A general discussion of the technical problems and difficulties encountered in this study is presented in Chapter III.

ABSTRACT

Allergen *Poa p* I was isolated from Kentucky Blue grass pollen extract by affinity chromatography with an anti-*Lol p* I murine monoclonal antibody (Mab) 290A-167 and was shown to consist of a 35.8kDa component with a pI of 6.4, designated as *Poa p* Ia (acidic) and a 33kDa component with a pI of 9.1, designated as *Poa p* Ib (basic). *Poa p* Ia appeared to be the major protein constituent of *Poa p* I on SDS-PAGE, and on Western immunoblot it also bound Mab 290A-167 to a greater extent than *Poa p* Ib. On the other hand, *Poa p* Ib was shown by Western immunoblot and autoradiographic analysis, to bind to a greater extent the IgE antibodies present in a pool of sera from grass allergic individuals. Therefore, *Poa p* Ib was considered as the major allergenic components of *Poa p* I.

The purified *Poa p* I was used to immunize mice for the preparation of Mabs. Five clones producing Mabs to *Poa p* I were selected. According to the antigenic specificities of both anti-*Poa p* I and anti-*Lol p* I Mabs to *Poa p* I, they could be classified into two groups: one recognized both *Poa p* Ia and *Poa p* Ib (anti-*Poa p* I Mabs 60 and 61, and anti-*Lol p* I Mab 290A-167); the other recognized *Poa p* Ib only (anti-*Poa p* I Mabs 62,63 and 64, and anti-*Lol p* I Mab 348A-6). The specificities of the Mabs were further resolved by comparing their respective abilities to inhibit the binding of I^{125}-*Poa p* I or I^{125}-*Lol p* I to the different Mabs prepared in the form of solid phase. These studies revealed that at least 4 distinct epitopes (designated as E_1,E_2,E_3 and E_4) were shared by both *Poa p* I and *Lol p* I. All 4

epitopes were present on *Poa p* Ia whereas only E_1 and E_3 were detected on *Poa p* Ib. E_1 was recognized by Mabs 60 and 61; E_2 by Mabs 62,63 and 64; E_3 by Mab 290A-167 and E_4 by Mab 348A-6. The observations that Mab 290A-167 inhibited significantly the binding of human IgE antibodies to *Poa p* I and *Lol p* I were interpreted to indicate that epitope E_3 may also constitute an allergenic site on both *Poa p* I and *Lol p* I. A schematic representation of a possible relationship of the epitopes on these allergens was constructed on the basis of the findings reported in this study.

INTRODUCTION

Allergic diseases

Allergic diseases are a diverse group of conditions which may be induced by a variety of environmental antigens (or allergens) which are present in pollens from grasses, weeds and trees; house dust mite (*Dermatophagoides pteronyssinus*); animal sources such as dander and feathers and foods (legumes, milk, eggs, seafood and nuts), etc (1,2). Allergies are mediated by IgE antibodies and are characterized by the immediate hypersensitivity reactions which follow exposure of the allergic individuals to the offending allergen(s) and may involve various organs and systems, i.e.

Eyes: lacrimation; itching; pain; redness; edema of the retina and the lids; vernal catarrh.

Nose: itching; sneezing; nasal obstruction; paroxysmal recurrent rhinorrhea; congestion.

Bronchi and lung: cough; wheezing; asphyxia.

Skin: erythema; pruritus; urticaria; itching.

Gastrointestinal tract: nausea; vomiting; intense diarrhea; stomach pain.

Genitourinary tract: occasionally essential hematuria; irritable bladder and cystitis (1).

These symptoms result from the actions of a host of pharmocologically active chemical mediators on the various organs and systems, causing smooth muscle constriction, bronchoconstriction, hypersecretion and mucosal oedema

which may thus lead to asthma, allergic rhinitis, gastroenteritis and hay fever (2,3). The term "hay fever" has been generally applied to allergic conditions caused by sensitization and subsequent exposure to allergens present in various pollens such as grasses, weeds and trees, and is characterized by seasonal and paroxysmal symptoms of rhinitis and conjunctivitis, i.e. profuse and paroxysmal rhinorrhea, sneezing, nasal obstruction, lacrimation and itching of the nose and the eyes (3).

Causes and mechanisms of allergic diseases

Allergens may possess different allergenic epitopes (certain natural sequences of amino acids in protein) which interact with the immune system to induce the production of IgE antibodies with the corresponding specificities for these epitopes. In order to initiate the process of antibody formation, the allergen needs first to be processed by an antigen presenting cell (APC) and presented on the surface of the APC in association with Ia molecules (or in humans, the DR molecules of the histocompatibility leukocyte antigen complex) (4-7). The allergen-Ia complex is recognized and bound by the receptors on T cells (8-10). Under the action of IL-1 produced by the APC, these T cells are activated to produce IL-4, IL-5 and IL-6 which then, accompanied with the native allergen, activate different clones of B cells which proliferate and differentiate to produce IgE antibodies with different specificities for the allergenic epitopes on the allergen (11-15).

The IgE antibodies have the property to bind to the mucosal and connective tissue mast cells and to the blood basophiles through the IgE receptors

on the surface of these target cells. This individual is now considered to be sensitized. On a subsequent exposure of the sensitized individual to the allergen, the cell-bound IgE antibodies become cross-linked through their binding to the allergenic epitopes on the allergen (16,17). As a consequence, the IgE receptors are perturbed and they transduce a signal(s) across the membrane which causes the target cells to undergo a process of degranulation and mediator release (18). Cross-linking of IgE receptors on the target cell causes the influx of calcium (Ca^{2+}) and the production of cyclic adenomonophosphate (cAMP), both act as a second messenger for the signal transduction in the cell (18). Increases in the levels of cAMP and the intracellular calcium initiate the formation of fusogens (e.g. active protein P, lysophosphatidylcholine, monoacylglycerol and free fatty acids etc). These fusogens cause fusion of the activated mast cell or basophil granule membranes into large granules which in turn fuse with the plasma membrane and cause extrusion of the granule contents into the extracellular fluid. These granules contain preformed mediators such as histamine, serotonin and heparin (18).

On the other hand, the bridging of IgE receptors on the target cells also induces the formation of arachidonic acid, which is further acted upon by 2 enzyme systems---lipoxygenases and cyclooxygenases---to respectively produce newly formed mediators, i.e. leukotrienes (formerly known as slow-reacting substance of anaphylaxis [SRS-A]) and prostaglandin D_2 (PGD_2) (19).

These preformed and newly formed mediators have vasoactive and inflam-

matory properties and act on adjacent smooth muscles and vascular endothelial cells to cause smooth muscle constriction, increase of vascular permeability and hypersecretion, which collectively result in the clinical symptoms of the allergy (18,19).

Allergens may also induce the production of blocking antibodies, which are predominantly of the IgG class in humans (20,21). Although these antibodies can bind to the antigenic epitopes on the allergen, they are unable to bind to or sensitise the target cells and therefore do not cause degranulation or mediator release. Moreover, once blocking antibodies become bound to the allergen, they may sterically hinder any further binding of IgE antibodies to the allergenic epitopes on the allergen. Hence, blocking antibodies may diminish mediator release and allergic inflammatory responses by interfering with the cross-linking process.

Grass pollen allergens and their characteristics

Rye grass (*Lolium perenne*) and Kentucky Blue grass (*Poa pratensis*) pollens are 2 major sources of aeroallergens that cause hay fever in North America and some areas of Europe. From a consideration of their physicochemical and biological properties, the allergens of Rye grass pollen have been classified into 4 major groups i.e. *Lol p* I,II,III and IV (Table 1). *Lol p* I (formerly designated as Rye I) has proven to be one of the major allergens and antigens in Rye grass pollen whereas *Lol p* II and III are regarded as minor allergens and antigens in terms of their relative allergenicity and antigenicity in both human and mouse cases (22-29). Re-

cently, *Lol p* IV (formerly designated as high molecular weight basic allergen or HMBA) was determined in our laboratory to be another major allergen of Rye grass pollen (Table 1) (30,31). Of these four major groups, *Lol p* I is the most important one since it can elicit skin reactions in 95% of grass-allergic patients (32). In order to determine the relationship between allergy to *Lol p* I and acute attacks of asthma, Pollart *et al* (33) has recently examined the IgE antibody which was obtained from sera of 59 patients presenting with acute asthma during a defined pollen season, to Rye grass pollen. The results demonstrated that 92% of the patients with asthma had >200 units of IgE antibody (~20ug of IgE/ml) to *Lol p* I compared to 14% of the control subjects (33). Also, Freidhoff *et al* (34) used purified *Lol p* I and *Lol p* II to characterize the longitudinal variation of both IgE and IgG antibody levels, as well as total serum IgE levels in grass-allergic subjects over a period of 13 months. They found that both specific IgE and IgG to *Lol p* I and *Lol p* II, as well as total IgE antibody levels declined toward a basal level just before, and increased just after, the grass-pollination season, returning to the same basal level just before the next grass-pollination season. They also found that grass-pollen counts were strongly correlated with symptom-medication scores for these subjects (34).

Lol p I is a glycoprotein which has been resolved into 4 isoallergens (I-A, I-B, I-C and I-D), of which I-B and I-C are the major components having a M.W. of 27-34kD with a carbohydrate content of ~5.4% and a pI of 5.15 (I-C)-5.25 (I-B). These isoallergens have no antigenic or allergenic cross-reactivity with other Rye grass pollen allergens, i.e. *Lol p* II, *Lol p*

ALLERGENS OF RYE GRASS POLLEN (*Lolium perennel*)

Allergens	Major Component	Mol. wt. (Daltons)	Isoelectric point	Carbohydrate (%)	Allergenicity[b]
Group I (Lol p I)	I-A, I-B, I-C, I-D	31-34 x 10^3 [a]	5.15 (I-C) 5.25 (I-B)	5.4	++++(I-B, I-C)
Group II (Lol p II)	II-A, II-B	11 x 10^3	Highly acidic	trace	++
Group III (Lol p III)	III-A, III-B	11 x 10^3	Basic	0	++
Group IV (Lol p IV)	HMBA	57 x 10^3	9.7	17	+++

a: Variations in the M.W. may be attributed to the different analytical methods used to determine the molecular weight.

b: The allergenicity of each group of allergen is evaluated by

(1) skin test in human
(2) in vitro histamine release assay from isolated allergic human leukocyte
(3) the ability of allergens to bind specific human IgE antibody or allergic human serum. (RAST)

Scale: ++++ highly allergenic

Table 1

III or *Lol p* IV (22-25,27). In order to identify the antigenic and allergenic epitopes on *Lol p* I, Bose *et al* (35) prepared twenty murine monoclonal anti-*Lol p* I antibodies and grouped them into five families in terms of their different epitope specificities. Hence, five distinct antigenic epitopes were detected on *Lol p* I since Mabs within one family did not compete with Mabs from other families for the binding to *Lol p* I. The results of the inhibition assays (testing the ability of each Mab to inhibit the binding of human IgE/IgG anti-*Lol p* I antibodies to *Lol p* I) indicated that these epitopes were also binding human IgE/IgG anti-*Lol p* I antibodies and may therefore be referred to as allergenic epitopes (35). Studies by Mourad *et al*, also using Mabs, have reveallled the presence of 4 different and non-overlapping, non-repetitive epitopes on *Lol p* I (36). The human IgE binding site on *Lol p* I was partially shared by each of these 4 epitopes (36). It is not presently known if any of the specificities of the epitopes identified in the studies of Bose *et al* (35) were related to those specificities reveallled in the studies of Mourad *et al* (36).

Studies by Freidhoff, Ansari and Marsh *et al* have shown that subjects allergic to *Lol p* II and *Lol p* III are usually also allergic to *Lol p* I, but not vice versa (37) and that immune responsiveness to all three allergens is significantly associated with the histocompatibility leukocyte antigen (HLA)-DR3 (38). Since there is no antigenic or allergenic cross-reactivity between *Lol p* I and *Lol p* II,III, the simultaneous allergic response to all three allergens can not be accounted for by suggesting that they share a common allergenic epitope which is recognized by an IgE antibody. The simul-

taneous allergic response to all three allergens was explained by suggesting that immune recognition of these three *Lol p* allergens may involve the interaction of an Ia molecule (HLA-DR3) with an Ia recognition site(s) common to all these three groups of allergens (28). However, this simultaneous allergic response to all three allergens may also be accounted for by suggesting that these three allergens may each contain different Ia recognition site(s) which may separately recognize different sites on the HLA-DR3 molecule. Given that an allergic individual may respond only to *Lol p* I, this allergen would appear to possess a unique Ia recognition site(s) not present in *Lol p* II and III (28).

The observation that individuals could be simultaneously allergic to different grass pollens may also be attributed to the fact that some grass pollens may share a common allergenic epitope(s) (i.e. specificity) which is recognized by an IgE antibody. Indeed, extensive immunological cross-reactivities have been found among various grass pollens (39-45). Based on their physical and immunochemical properties, antigens similar to or cross-reactive with *Lol p* I (or group I type antigens) have been found in other grass pollens, including Timothy and Kentucky Blue grass pollens (39,41,44-46). Studies comparing related proteins obtained from a variety of sources suggest that their degree of cross-reactivity is directly related to the homology of their amino acid sequences (47). Therefore, it may be suggested that the group I type antigens share a common structure or amino acid sequence which constitutes the common antigenic or allergenic epitopes capable of binding specifically to a given antibody. This is partially sup-

ported by Mourad's recent study (36) in which it was demonstrated that three different anti-*Lol p* I Mabs (290A-167, 348A-6 and 539A-6) and one anti-*Dac g* I Mab (P3B2) were all capable of bindng to *Dac g* I (the major allergen of *Dactylis glomerata* pollen) and *Lol p* I. Further studies showed that the four Mabs were directed against four different and non-overlapping, non-repetitive epitoeps present on both allergens (36). They concluded that *Dac g* I and *Lol p* I share four identical epitopes in addition to their similar physicochemical characteristics, such as pI and M.W. (36). Thus, identification of the antigenic or allergenic epitopes may help to elucidate the basis of the cross-reactivity.

Requirements for purified, well-characterized allergens

Crude pollen extracts, commonly used in immunotherapy, are heterogeneous mixtures of allergenic and non-allergenic components. Thus, treatment with a crude extract can induce, in some patients, sensitivity to some pollen constituents to which they were not originally sensitive (48-50). Purified and well characterized allergens, therefore, are essential for producing modified allergens or developing other rational approaches for immunotherapy. Moreover, blocking antibodies (IgG) are present in very low titer in most, if not all, hay fever patients, and they may reach a very high titer following immunotherapy. The increase in the level of IgG antibodies is usually accompanied by a decrease in the level of IgE antibodies. The isolation and purification of the allergens and identification of their epitopes may, therefore, facilitate the preparation of the tolerogenic form

of the allergens which may be useful for treating allergic patients by suppressing the production of the IgE antibody or by stimulating the production of blocking antibodies. Given the extensive cross-reactivity of many related allergens, it is postulated that the tolerogenic form of a given allergen could be used as a vaccine to suppress the formation of IgE antibodies not only to the homologous allergen but also to other cross-reactive antigens (or allergens) from the related grass pollens.

The conventional physicochemical techniques which are used for the isolation and purification of allergens from a complex mixture of components usually involve a combination of several methods, may still be unable to separate components with similar physicochemical properties. The availability of monoclonal antibodies (Mab) to a variety of allergens (31,39,41,44-46,51,52) has proven useful not only for the detection of specific determinants on allergens and for comparing antigenic relationships, but also for the preparation of reverse immunosorbents for the direct isolation of individual components from a complex mixture (31,39,51,52). Clearly, monoclonal antibodies can serve to isolate not only the homologous allergens, but also the cross-reactive components from the related grass pollens.

Scope of the present study

In studies by Mourad *et al*, monoclonal antibodies were prepared to *Lol p* I. The hybridoma cells were obtained by fusing myeloma cells (SP2/0-Ag14) and spleen B cells of mice immunized with *Lol p* I which has a major component of 34kD and was obtained from NIH (39). Three clones, 290A-167 (IgG_{2b}),

348A-6 (IgG_1) and 539A-6 (IgG_{2a}), were obtained. Mabs 290A-167 and 539A-6 were also found to be cross-reactive with components present in Kentucky Blue grass pollen, of which Mab 290A-167 recognized a major component of 36kD and a minor component of 33kD and Mab 539A-6 also recognized these two components as well as additional components with M.W. of 18-22kD (39).

Advantage was taken of the observations that Kentucky Blue grass pollen contained components which cross-reacted with the *Lol p* I allergens and that monoclonal antibodies were available that recognized the *Poa p* I allergens. The objectives of the present study were to: a) isolate the cross-reactive *Poa p* I antigens from Kentucky Blue grass pollen using a Mab to *Lol p* I (Mab 290A-167), b) use the purified antigen to immunize mice and to produce Mabs to *Poa p* I, c) characterize *Poa p* I both immunologically and physicochemically and d) compare the antigenic and allergenic relationships of the *Poa p* I antigen with the *Lol p* I antigen.

Chapter I

Isolation and identification of *Poa p* I and the production of monoclonal anti-*Poa p* I antibodies.

INTRODUCTION

Rye grass pollen allergen *Lol p* I (Rye I) is the most extensively studied group I type allergen. Nevertheless, based on their similar physical and immunochemical properties to *Lol p* I, group-I type antigens have also been found in other grass pollens, including Kentucky Blue grass pollen (39,41,44) which is one of the major allergy causing agents in the Prairies and western regions of North America and also in various areas of Europe. In this chapter, we described how a monoclonal anti-*Lol p* I antibody, Mab 290A-167 was utilized for the isolation and characterization of the cross-reactive *Poa p* I antigen from Kentucky Blue grass pollen. With this Mab, we isolated the cross-reactive and analogous components from KBG, named *Poa p* I. *Poa p* I was also found to be an allergen in terms of its capability of binding human IgE antibodies. The purified *Poa p* I antigens were then used for the immunization of mice for the production of monoclonal antibodies to *Poa p* I.

ABSTRACT

The *Poa p* I allergens were isolated from the retentate fraction of a dialysed preparation of an aqueous extract of Kentucky Blue grass pollen (KBG-R) by means of a reverse immunosorbent prepared with a murine anti-*Lol p* I monoclonal antibody, Mab 290A-167. By SDS-PAGE and preparative IEF, *Poa p* I was found to consist of a 35.8kDa component with a pI of 6.4 and a 33kDa component with a pI of 9.1 and designated as *Poa p* Ia (acidic) and *Poa p* Ib (basic) respectively. The relative protein content of these components was estimated from the intensity of the stained bands following the SDS-PAGE. Thus, *Poa p* Ia appeared to be the major protein constituent and on Western immunoblot it also bound the monoclonal antibody to a greater extent than *Poa p* Ib. On the other hand, *Poa p* Ib was shown by Western immunoblot and autoradiographic analysis, to bind to a greater extent the IgE antibodies present in a pool of sera from grass allergic individuals. Therefore, *Poa p* Ib was considered as the major allergenic component of *Poa p* I.

The purified *Poa p* I was used to immunize mice for the preparation of Mabs. Five clones producing Mabs to *Poa p* I were selected for propagation in the form of ascities in mice. The resulting Mabs could be classified into two groups; one which bound both *Poa p* Ia and *Poa p* Ib (Mabs 60,61); the other which bound *Poa p* Ia only (Mabs 62,63,64).

MATERIALS AND METHODS

Preparation of KBG-R

The non-dialysable components of Kentucky Blue grass (*Poa pratensis*) (Hollister-Stier Laboratory, Mississauga, Ontario, Canada) were prepared by extracting ether defatted pollen with H_2O followed by dialysis through Spectrapor membrane tubing (mol wt. cut off = 6 to 8kDa, Spectrum Medical Industries, Inc., Los Angeles, CA, USA). The retentate fraction was designated as KBG-R.

Production and Purification of Monoclonal anti-Lol p I Antibodies, Mabs 290A-167 and 348A-6.*

The monoclonal antibodies, Mabs 290A-167 and 348A-6 were two of several anti-*Lol p* I (anti-Rye I) monoclonal antibodies recently described by Mourad *et al* (39). Briefly, BALB/c female mice were immunized with purified *Lol p* I antigens (obtained from NIH, Bethesda, MD, USA) emulsified in Freund's complete adjuvant. The spleen cells from the immunized mice were fused with SP2/0-Ag14 myeloma cells. Monoclonal cell lines were produced by the limiting dilution method. Clones of hybridoma cells secreting antibody to *Lol p* I were expanded in vitro and then propagated in the peritoneum of BALB/c female mice. The ascitic fluid containing the Mab was collected and dialyzed through Spectrapor membrane tubing (mol wt. cut off = 6 to 8kDa) against buffer A (0.025M Triethanolamine-HCl, pH 7.7) at 4°C overnight. The

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*Mabs 290A-167 and 348A-6 were prepared and kindly provided by Mourad *et al*.

nondialyzable components were centrifuged at 20,200g for 10 min and the supernatant was filtered through a sterile millipore membrane (0.45um pore size, Millex-HA, Millipore Corp. Bedford, MA, USA). The filtered material was then fractionated by Fast Protein Liquid Chromatography (FPLC) using a Mono Q (HR 5/5) anion exchange column (Pharmacia Fine Chemicals AB, Montreal, Que., Canada) equilibrated with buffer A. The proteins were eluted by applying a linear gradient of buffer B (buffer A containing 0.354M NaCl). The fractions containing antibody activity were pooled. The isotypicity of the Mabs was determined by ELISA as outlined in the section on "ELISA" and confirmed to be IgG_{2b} (*k*) for 290A-167 and IgG_1 (*k*) for 348A-6 (39).

Allergic Human Serum

Serum was obtained in Manitoba during 1976 from six patients highly allergic to grass pollen. The sera were pooled, stored at -20°C and have been used in this laboratory for the long-term studies on the allergenic constituents of Kentucky Blue grass pollen.

Isolation of Poa p I

(a) *Preparation of Reverse Immunosorbent*

The reverse immunosorbent was prepared as described previously (51) with the following modifications. CNBr-activated Sepharose 4B (8.6g) (Pharmacia) was washed with 30ml of ice-cold aqueous solution of HCl (10^{-3}M). Eighteen ml of the Mab 290A-167 preparation at a concentration of

3.42mg/ml, was mixed with the gel in a stoppered plastic centrifuge tube. The gel suspension was rotated end-over-end for 1 hr. The excess antibody was washed away with coupling buffer. From a measurement of the optical density of the combined washings, it was estimated that 2.8mg of the Mab was coupled to 1ml of the gel. Any remaining active groups were blocked by incubating the gel with 0.1M Tris-HCl buffer, pH 8. The gel was alternately washed with acidic and basic buffers according to the manufacturer's direction. The reverse immunosorbent, thus obtained, was then washed with 4M guanidine-HCl buffer, pH 3.8, followed by PBS.

(b) *Affinity Chromatography*

A solution of KBG-R (50mg dissolved in 30ml of PBS) was applied onto the reverse immunosorbent column. The column was then thoroughly washed with PBS. The material which had bound to the immunosorbent column was eluted with of 4M guanidine-HCl containing 0.25M sodium acetate, pH 3.8. The eluate was immediately neutralized with 2M Tris-HCl, pH 8.5. The eluted material, designated as *Poa p* I, was dialysed against an aqueous solution of 10^{-3}M ammonium bicarbonate at 4°C overnight.

(c) *Measurement of Protein Content*

The protein concentration of solutions of *Poa p* I was determined by the method of Lowry (55). The protein concentration of solutions of the purified Mab was determined from the OD at 280nm (using ${}_{280}E^{1\%}=13.8$ for mouse IgG and ${}_{280}E^{1\%}=11.85$ for mouse IgM).

(d) *Polyacrylamide Gel Electrophoresis*

-*SDS-PAGE*: This was performed in the protein slab cell apparatus (Bio-Rad; Richmond, CA) with 1.5 mm thick polyacrylamide gels (12%) utilizing the Laemmli buffer system (56). The sample was incubated in sample buffer overnight at room temp prior to electrophoresis. The gel was stained with Coomassie Brilliant blue R250 (Kodak, Eastman Kodak Co. Rochester, N.Y. USA) according to the method of Fairbanks *et al.* (57). Low molecular weight protein standards (Bio-Rad) were used to calibrate the gels for the determination of the relative molecular weights (M*r*) of the electrophoresed components.

-*Western blot*: Proteins (KBG-R or *Poa p* I) separated by SDS-PAGE were electrophoretically transferred onto nitrocellulose membrane (58). To block its ability to further bind protein, the membrane was incubated overnight with gelatin (3%) in TBS (25mM Tris-HCl, 0.5M NaCl, pH 8.3).

-*Immunoblot*: Detection of proteins on the nitrocellulose was achieved by immunoblot as follows. After blocking, the membrane was incubated with a solution of purified anti-*Poa p* I or anti-*Lol p* I Mabs (8.5ug/ml in TBS containing gelatin(1%)) at room temp overnight. The membrane was washed with TBS, then incubated with goat-anti-mouse IgG horseradish peroxidase conjugate (Bio-Rad) (diluted 3000-fold with gelatin (1%) in TBS) at room temp for 1 hr. After washing the membrane with TBS, the colour reaction was developed by adding a freshly prepared mixture of Horseradish Peroxidase colour development reagent, 4-chloro-1-napthol (Bio-Rad) and 30% H_2O_2.

-*Autoradiography*: Following the separation of *Poa p* I antigens by SDS-PAGE and transfer to nitrocellulose, the membrane was immersed in a solution of the human allergic serum pool (diluted 5-fold with TBS containing 1% gelatin) at room temp for 40 hrs. The membrane was washed in TBS, then incubated with ^{125}I-labelled murine monoclonal anti-human IgE antibody ($6x10^6$ cpm in 60ml of TBS containing gelatin(1%)) overnight. (This antibody, clone # CIA/E/4.15 specific for human IgE was generously supplied by Dr. A. Saxon, UCLA, Los Angeles, CA). The membrane was washed with PBS containing Tween 20 (1%), and then air dried and exposed to X-ray film (Kodak, Rochester, N.Y., USA) at -70°C for 4 hrs. The X-ray film was developed and fixed according to the manufacturer's directions.

Production and Purification of Monoclonal anti-Poa p I Antibodies

Female BALB/c mice were immunized intraperitoneally (i.p.) with 25ug of affinity purified *Poa p* I emulsified in complete Freund's adjuvant. Booster injections were administered on days 21 and 36 with the same amount of *Poa p* I in saline. On day 39, the immune spleen cells were fused with NS1 myeloma cells using polyethylene glycol solution as previously described (53). The cell pellet obtained was resuspended in RPMI complete medium and aliquoted in 24-well Costar plates. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and were maintained by alternate addition or replacement of HAT medium which contains hypoxanthine, aminorpterin and thymidine (HAT). Four fusions were performed, generating a total of 384 separate primary cultures. Aliquotes of the culture supernatants were

assayed for antibody activity by ELISA. Based on the results of ELISA, thirty six cultures were selected and then expanded and retested for antibody production prior to cloning. The monoclonal cell lines were prepared by the limiting cell dilution method. The specificity of the monoclonal antibodies for *Poa p* I was further examined by Western immunoblot. Five monoclonal cell lines (250.38,B4.2/150 (Mab 60); 250.38,B4.2/1 (Mab 61); 249.38,C1.2/128 (Mab 62); 251.38,C3.5/36 (Mab 63) and 251.38,C3.5/128 (Mab 64)) selected on the basis of their specific reactivity to *Poa p* I were maintained in flasks for mass culture and then propagated in the peritoneum of BALB/c female mice (a suspension of $4\text{-}5 \times 10^6$ viable hybrid cells in RPMI complete medium was injected i.p. into each mouse). The ascitic fluid was harvested 10-15 days later. After precipitation of the immunoglobulins in the ascitic fluids with a saturated solution of ammonium sulphate (33% of saturation), the Mabs were purified by gel filtration on AcA-44 Ultrogel. The fractions were tested by ELISA as described below and pooled on the basis of their anti-*Poa p* I activity. The purity of the Mabs was tested by SDS-PAGE.

Mab 27 was produced by immunization of mice with KBG-R as described previously (54) and purified by FPLC system on a Mono Q (HR 5/5) anion exchange column as mentioned before.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed with the 96 well-EIA plate (cat #3590, Costar, Cambridge, MA, USA) as described elsewhere (51) with the following

modifications. The plates were coated with antigen (KBG-R or *Poa p* I, 10ug/well) in coating buffer (1.59g of Na_2CO_3, 2.93g of $NaHCO_3$ and 0.2g of NaN_3 made up to 1 liter with distilled water, pH 9.6) at 4°C overnight, and blocked with bovine serum albumin (BSA) 1% in PBS (0.54g KH_2PO_4, 7.75g NaCl, 0.5g NaN_3, H_2O to make 1L, pH 7.2). For assaying the antibody activity of the FPLC and AcA-44 gel filtration fractions, the KBG-R coated plates were incubated with the various fractions diluted 10-fold with assay buffer (AB, PBS containing BSA(0.5%), Tween 20 (1%)) for 1 hr. After washing the plates with PBS containing Tween 20 (1%), a conjugate of rabbit anti-mouse IgG alkaline phosphatase (Sigma chemical Corp. St. Louis, MO, USA) diluted 1000-fold with AB was added and incubated for 1 hr. Following washings, the enzyme substrate, p-nitrophenolphosphate (1mg/ml) was added and the colour reaction was developed for 40 min, then stopped by adding 3M NaOH. The optical density was measured at 410nm using the Microplate Reader (Model MR600, Dynatech Laboratories Inc. Alexandria, VA, USA.).

For assaying the antigenic activity of purified *Poa p* I antigens, the Mab 290A-167 preparation was serially diluted with AB and then added to the *Poa p* I antigen coated plates and incubated overnight. The ELISA was continued as described above.

For determining the allergenic activity of *Poa p* I by ELISA, the allergic human serum pool was serially diluted, added to *Poa p* I coated plates, and incubated overnight. After washing, a conjugate of goat anti-human IgE alkaline phosphatase (Sigma) diluted 1000-fold with AB was added and incubated overnight. The ELISA was then continued as described above.

The isotypicity of the Mabs was determined by ELISA using the Mono-Ab ID ELISA kit (Zymed Lab. San Francisco, CA) according to the manufacture's instructions. Briefly, EIA plates were coated with *Poa p* I (0.5ug/well), and blocked with 1% BSA in PBS for 2 h. A solution of Mab, 0.5ug in 100ul of assay buffer (0.5% BSA in PBS containing 0.1% Tween 20) was added to each well and incubated overnight. After washing the plates with PBS-Tween solution, rabbit antibodies specific for one of the Ig classes or subclasses was added to the wells, kept for 1 h at 37°C and then washed. Goat anti-rabbit Ig alkaline phosphatase conjugate was added to the wells and kept for 1 h at 37°C. Following washings with the PBS-Tween solution, a solution of the substrate (1mg/ml of p-nitrophenyl phosphate in 10% diethanolamine buffer, pH:9.8) was added. The colour reaction was developed for 30 min and stopped by the addition of 3M NaOH. The OD was measured at 410 nm using the Microplate Reader (Model MR 600, Dynatech Lab. Inc. Alexandria,VA).

Preparative Isoelectrofocusing

One hundred mg of KBG-R was fractionated by preparative IEF as described previously (51). Briefly, Ultrodex (6g) (LKB-Products AB, Stockholm-Bromma, Sweden) was swollen in a solution (150ml) of KBG-R (100mg) and the ampholyte buffer (7.5ml, Ampholine, pH range 3.5-10,LKB) and poured onto a horizontal glass tray (11x30x0.8 cm, Bio-Rad).

The isoelectrofocusing was carried out at 4°C for 96 hrs. The gel was then divided into 38 equal sections with the aid of a gel divider (Bio-Rad). Each gel section was eluted with water (5ml). The absorbance, at 280nm, and

the pH of each eluted fraction were measured. The ability of each fraction to bind the anti-*Poa p* I Mab 61 and Mab 62 was examined by ELISA. The specificity of these Mabs is described in "Results".

RESULTS

The allergenic and antigenic properties of *Poa p* I isolated from KBG-R with the Mab 290A-167 immunosorbent were evaluated by ELISA in terms of the ability of *Poa p* I to bind IgE antibodies present in the pool of human grass-allergic sera and Mab 290A-167, respectively. The results (Fig.1,bottom panel) clearly showed that the *Poa p* I antigen was capable of binding the human IgE antibodies. *Poa p* I also bound Mab 290A-167 (Fig.1,top panel). The specificity of the assay with Mab 290A-167 was demonstrated by the observation that another murine monoclonal antibody, Mab 27 (IgG_1,*k*), produced against KBG-R (51,54) and possessing a specificity unrelated to Mab 290A-167, did not bind to the *Poa p* I antigen. Moreover, the antigenic components of *Poa p* I which could be detected by Western and immunoblot analysis (Fig.5) with Mab 290A-167 failed to bind Mab 27 on the immunoblot (data not shown). By means of SDS-PAGE, using either reducing or non-reducing conditions, the affinity purified *Poa p* I was shown to be composed of two components with M*r* of 35.8kDa and 33kDa (Fig.2b), and they have been provisionally designated as *Poa p* Ia (acidic) and *Poa p* Ib (basic), respectively (see Chapter III). Based on their intensity of staining, it would appear that *Poa p* Ia was the major protein component of the *Poa p* I antigen. *Poa p* Ia was also shown to be one of the major protein components of KBG-R (Fig.2a).

Poa p I was used to immunize the mice to produce monoclonal antibodies (Mab). Based on their specificity, two groups of anti-*Poa p* I Mabs were ob-

Fig.1 <u>Antigenic and allergenic activities of *Poa p* I antigens as demonstrated by ELISA</u>. Plates were coated with *Poa p* I (10ug/well). Top panel; serial dilutions of purified Mab 290A-167 (0.85mg/ml) (△-△) or Mab 27 in ascites form (□-□) was added to the antigen-coated wells. A conjugate of rabbit anti-mouse IgG and alkaline phosphatase was used as the second antibody. Bottom panel; serial dilutions of an allergic serum pool were added to antigen-coated wells (●-●). Goat anti-human IgE conjugated to alkaline phosphatase was used as the second antibody.

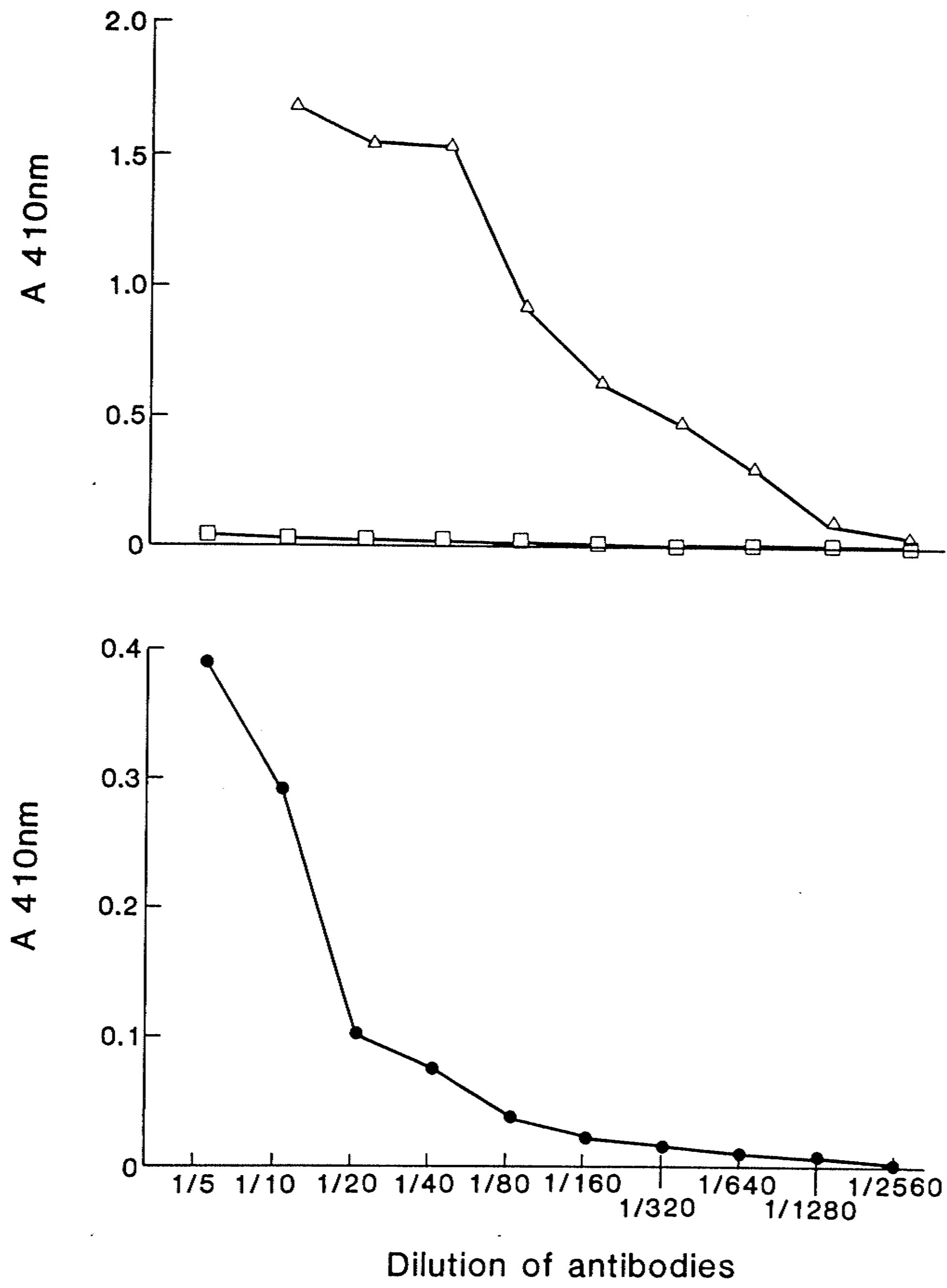

Fig.1

Fig.2 <u>SDS-PAGE of KBG-R and *Poa p* I stained with Coomassie Brilliant blue</u>.

a: KBG-R, 100ug. b: *Poa p* I, 40ug. c: Protein markers, 2.5ug each.

All samples were reduced with 2-Mercaptoethanol prior to electrophoresis.

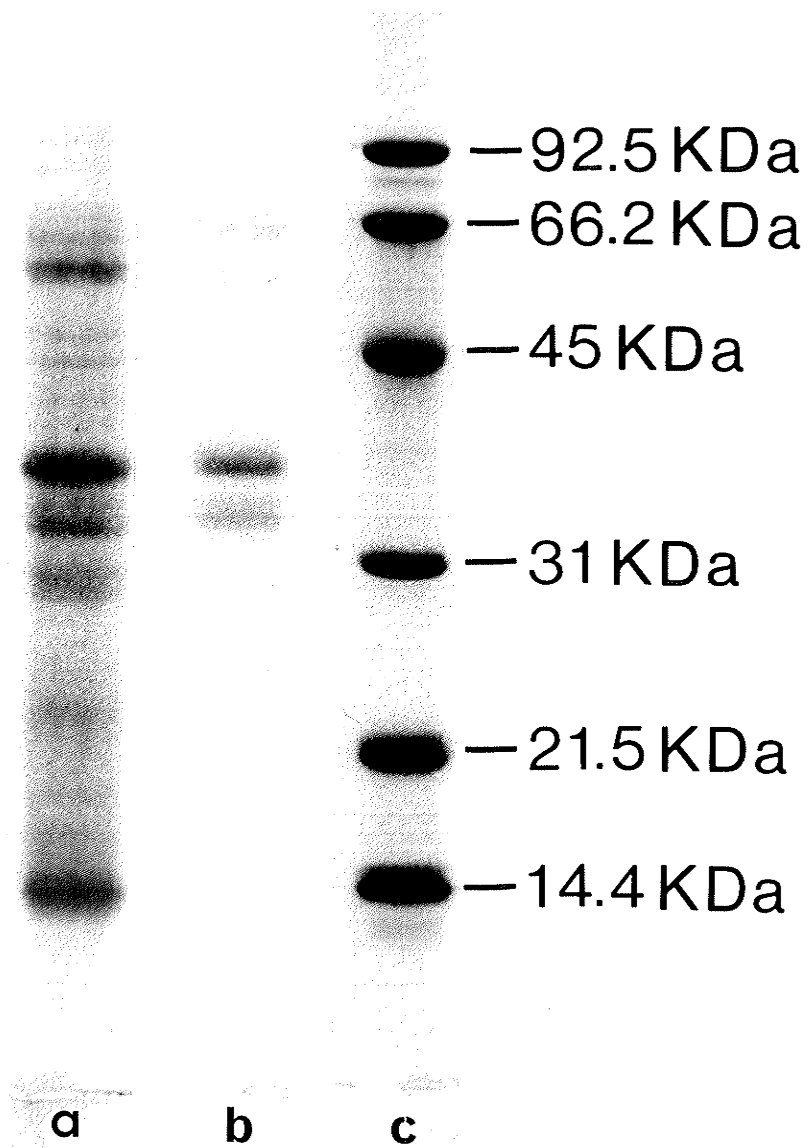

Fig.2

tained. One group bound both *Poa p* Ia and *Poa p* Ib, whereas the other group bound only *Poa p* Ia (Fig.3). Surprisingly, none of the clones secreting antibody to *Poa p* I was directed exclusively to *Poa p* Ib. These results were interpreted to indicate that *Poa p* Ia is the major immunogenic component of *Poa p* I. Among these Mabs, five clones were selected and designated as Mab 60, Mab 61, Mab 62, Mab 63 and Mab 64. Mabs 60 and 61 were of the IgG_1 isotype with *k* chains while Mabs 62,63 and 64 were of the IgM isotype with *k* chains. By SDS-PAGE and Immunoblot analyses, it was found that both Mabs 60 and 61 recognized *Poa p* Ia and *Poa p* Ib whereas Mabs 62,63 and 64 recognized only *Poa p* Ia (Fig.3). In addition, the anti-*Lol p* I monoclonal antibodies, Mabs 290A-167 (IgG_{2b},*k*) bound both *Poa p* Ia and *Poa p* Ib whereas Mab 348A-6 (IgG_1,*k*) bound to *Poa p* Ia only (Fig.3).

The pI values of the components of *Poa p* I were determined by preparative IEF of KBG-R. (KBG-R was used due to a lack of a sufficient amount of the purified *Poa p* I.) The separated fractions were analysed by ELISA using Mab 61 which recognized both *Poa p* Ia and *Poa p* Ib, and Mab 62 which recognized *Poa p* Ia (Fig.4). The results revealed that the components of KBG-R capable of binding Mab 61 were associated with well separated acidic (pI~6.4) and basic (pI~9.1) fractions. On the other hand, the components of KBG-R capable of binding Mab 62 were associated only with the acidic fractions. Since Mab 61 recognizes both *Poa p* Ia and *Poa p* Ib and Mab 62 recognizes only *Poa p* Ia, it was concluded that *Poa p* Ia was acidic and *Poa p* Ib was basic. This conclusion was confirmed on further examination of the acidic and the basic fractions by SDS-PAGE and Western immunoblot analysis

Fig.3. <u>SDS-PAGE, Western and Immunoblot analyses to examine the specificity of the Mabs for *Poa p* I</u>. *Poa p* I was separated by SDS-PAGE and transfered onto nitrocellulose membrane. The immunoblots were made separately with each of the Mabs. Goat anti-mouse IgG(H+L) horseradish peroxidase conjugate was used as the developing antibody.

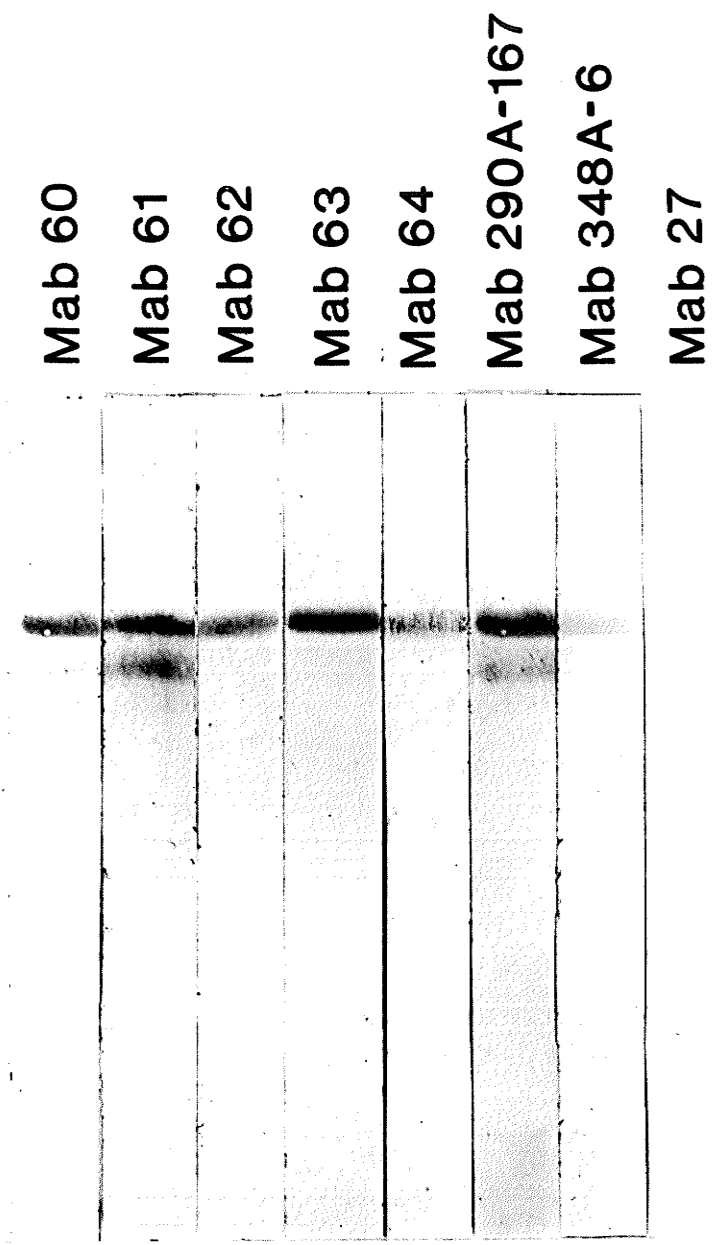

Fig.3

Fig.4 Preparative isoelectrofocusing of KBG-R (100mg) on Ultrodex (Ampholine: pH 3.5-10; 96h). The extent to which the fractions bound Mab 61 and Mab 62, by ELISA, are indicated with the solid and dashed lines, respectively.

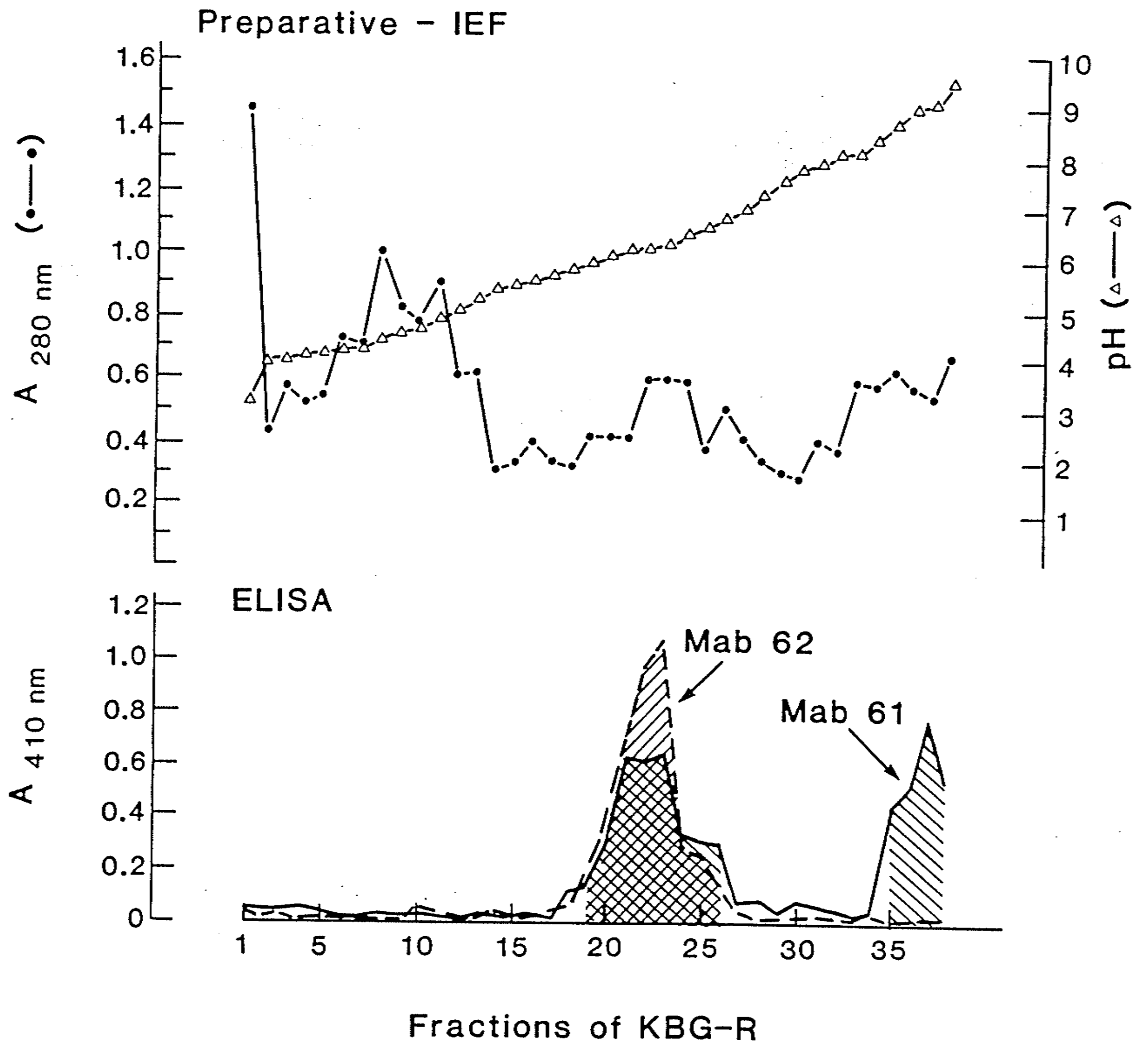

Fig.4

employing Mab 61 and Mab 62 (data not shown). Also, similar results were obtained by using anti-*Lol p* I monoclonal antibodies* (Mabs 6,8,11,20), in which Mabs 6 and 11 bound both *Poa p* Ia and *Poa p* Ib while Mabs 8 and 20 bound *Poa p* Ia only (data not shown).

For further analyses of *Poa p* I, combinations of SDS-PAGE, Western immunoblot and autoradiography techniques were used. Both components of *Poa p* I separated on SDS-PAGE were capable of binding Mab 290A-167 (Fig.5a). As expected, these two components were also detected in KBG-R (Fig.5b). The major protein-staining component, *Poa p* Ia (35.8kDa, cf Fig.2b) strongly bound Mab 290A-167 (Fig.3,5a). By contrast, the minor protein-staining component, *Poa p* Ib (33kDa, cf Fig.2b) reacted more strongly with human IgE antibodies as revealed by autoradiography (Fig.6a) and is therefore referred to as the major allergenic component. At least four radiostaining bands were detected on the autoradiograph in the analysis of KBG-R (Fig.6b). These results indicated that KBG-R possessed at least four groups of allergenic components which differed in their sizes. Clearly, *Poa p* Ib is one of the major allergens of KBG-R.

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*Monoclonal antibodies against *Lol p* I (Mabs 6,8,11 and 20) were produced by Dr. Bose (35) in our Hybridoma Unit of the MRC Group for Allergy Research, using *Lol p* I antigen that was kindly supplied by Dr. D. Marsh, Dept. of Medicine, Johns Hopkins University, Baltimore, MD, USA.

Fig.5 SDS-PAGE and Western immunoblot analyses of KBG-R and *Poa p I*. The immunoblot was developed with Mab 290-A-167. a: *Poa p* I, 40ug. b: KBG-R,150ug.

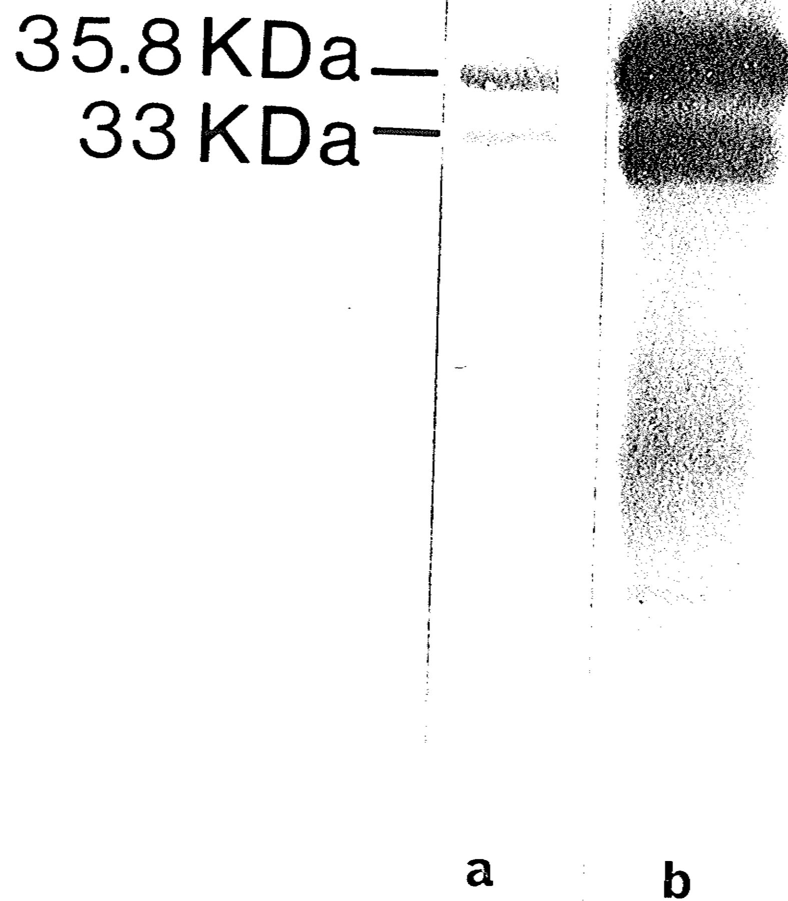

Fig.5

Fig.6 <u>Detection of allergenic components in *Poa p* I and KBG-R by SDS-PAGE, Western immunoblot and autoradiography</u>. Components separated by SDS-PAGE were transferred onto the nitrocellulose membrane. The membrane was then incubated with a human allergic serum pool. The components which bound human IgE antibody were detected by employing an ^{125}I-labelled murine monoclonal anti-human IgE antibody as described in "Methods". a; *Poa p* I, 40ug. b; KBG-R, 100ug.

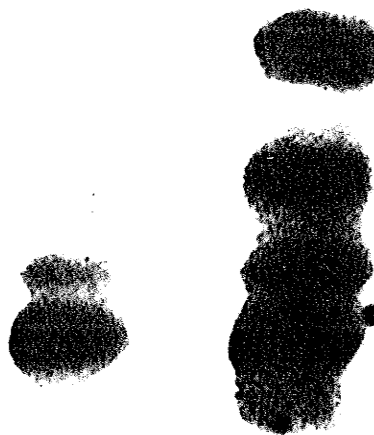

Fig.6

DISCUSSION

A murine monoclonal antibody, Mab 290A-167 which had been prepared against the *Lol p* I antigens of Rye grass pollen (39), was used to identify the cross-reactive *Poa p* I antigen of Kentucky Bluegrass pollen. Whereas *Lol p* I has been reported (39) to consist of a major component with a *Mr* of 34kDa and a minor component of 23kDa, the analysis of the retentate fraction of the aqueous extract of KBG pollen by a combination of SDS-PAGE and Western immunoblot with Mab 290A-167 detected two components with *Mr* of 35.8kDa and 33kDa, which were designated as *Poa p* Ia and *Poa p* Ib, respectively. These two components of *Poa p* I were isolated directly from KBG-R by means of a reverse immunosorbent prepared with Mab 290A-167. The 35.8kDa component (*Poa p* Ia) stained more heavily than the 33kDa (*Poa p* Ib) and was therefore regarded as having the higher protein content. Western immunoblot analysis revealed, as expected, that both components bound Mab 290A-167. Since *Poa p* Ia bound Mab 290A-167 to a greater extent than *Poa p* Ib, it is suggested that the density and/or the total number of epitopes recognized by Mab 290A-167 is greater in *Poa p* Ia than in *Poa p* Ib. Also, the production of monoclonal anti-*Poa p* I antibodies indicated that *Poa p* Ia is the major immunogenic component of *Poa p* I since none of the clones secreting antibody to *Poa p* I was directed exclusively to *Poa p* Ib. However, it appeared that *Poa p* Ib had a greater capacity to bind IgE antibodies of sera from grass allergic individuals. Therefore, it is suggested that the density and/or the total number of epitopes recognized collectively by the human IgE is greater

for *Poa p* Ib than for *Poa p* Ia. Moreover, the differences in the specificities of the anti-*Poa p* I and anti-*Lol p* I Mabs enabled us to identify the pI of both *Poa p* Ia (~6.4) and *Poa p* Ib (~9.1) as shown in Fig.4, and indicated that , on the other hand, at least 2 antigenic epitopes (E_2 for Mabs 62,63 and 64 and E_4 for Mab 348A-6) are present on *Poa p* Ia but not on *Poa p* Ib. The later statement was based on the findings that none of the Mabs 62,63,64 and 348A-6 was able to bind *Poa p* Ib by Western immunoblot (Fig.3) and that Mab 348A-6 had different specificity for *Poa p* I from that of Mabs 62,63 and 64 described in Chapter II.

One of our recent findings demonstrated that a murine monoclonal antibody, Mab 27, produced by immunization of mice with KBG-R, had the ability to bind strongly a 30kDa component of the Kentucky Blue grass pollen (51). Therefore, the possibility that Mab 27 could bind to *Poa p* I was considered. However, the results of the present study revealed that neither *Poa p* Ia nor *Poa p* Ib was able to bind Mab 27. It was concluded that the specificity of Mab 27 was different from Mab 290A-167 and the other Mabs used in the present study.

Chapter II

Mapping of epitopes on *Poa p* I and *Lol p* I with monoclonal antibodies

INTRODUCTION

An elucidation of the structural features and an analysis of the relationship between the allergenic and antigenic epitopes on a given allergen is a very important goal in allergy research. Knowledge of the repertoire of allergenic specificities recognized by any one individual should facilitate the diagnosis of the breadth of the allergic response. Moreover, the identification of the entire repertoire of allergenic specificities may be useful also in evaluating the potency of allergenic extracts used for immunotherapy. Moreover, it may lead to developing more effective methods of potential therapy of the allergic patients by, for example, preferentially stimulating the production of blocking antibodies with the appropriate synthetic antigenic epitopes.

The studies described in the previous chapter have shown that *Poa p* I is one of the major allergens in KBG allergen and consists of 2 components, *Poa p* Ia (35.8kDa) and *Poa p* Ib (33kDa), which differ in their antigenic and allergenic activities. In addition, *Poa p* I was found in our studies (60) and those of others (39,41,44) to be antigenically cross-reactive with the *Lol p* I allergen of Rye grass pollen. The availability of monoclonal antibodies with the desired specificities might provide a means to investigate the molecular basis of this cross-reactivity.

The study described in this chapter was undertaken to establish the nature of the cross-reactivities of *Poa p* I and *Lol p* I using Mabs prepared either against *Poa p* I or *Lol p* I and allergic human sera. It was found that *Poa p* I and *Lol p* I possessed similar antigenic and allergenic epitopes.

ABSTRACT

Allergen *Poa p* I isolated from KBG pollen extract by affinity chromatography with an anti-*Lol p* I murine monoclonal antibody (Mab) 290A-167 was previously shown to consist of a 35.8kD component with a pI of 6.4, designated as *Poa p* Ia and a 33kD component with a pI of 9.1, designated as *Poa p* Ib. The present study reports on the comparative antigenic analyses of these two components, using Mabs produced separately against *Poa p* I and *Lol p* I. Thus, anti-*Poa p* I Mabs 60 and 61 and anti-*Lol p* I Mab 290A-167 recognized *Poa p* Ia and *Poa p* Ib whereas anti-*Poa p* I Mabs 62,63 and 64 and anti-*Lol p* I Mab 348A-6 recognized only *Poa p* Ia. The specificities of the Mabs were further resolved by comparing their respective abilities to inhibit the binding of I^{125}-*Poa p* I or I^{125}-*Lol p* I to the different Mabs prepared in the form of solid phase. These studies revealed that at least 4 distinct epitopes (designated as E_1,E_2,E_3 and E_4) were shared by both *Poa p* I and *Lol p* I. All 4 epitopes were present on *Poa p* Ia whereas only E_1 and E_3 were detected on *Poa p* Ib. E_1 was recognized by Mabs 60 and 61; E_2 by Mabs 62,63 and 64; E_3 by Mab 290A-167 and E_4 by Mab 348A-6. The observations that Mab 290A-167 inhibited significantly the binding of human IgE antibodies to *Poa p* I and *Lol p* I were interpreted to indicate that epitope E_3 may also constitute an allergenic site on both *Poa p* I and *Lol p* I. A schematic representation of a possible relationship of the epitopes on these allergens was constructed on the basis of the findings reported in this chapter.

MATERIALS AND METHODS

Isolation of Poa p I

Poa p I was isolated from the retentate fraction of Kentucky Blue grass (KBG-R) pollen by means of a reversed immunosorbent prepared with a murine anti-*Lol p* I monoclonal antibody, Mab 290A-167 as described in Chapter I. The purity of *Poa p* I was tested by SDS-PAGE.

Allergic human serum

Sera were obtained in Manitoba from 3 individuals (JY,DB and BW) highly allergic to grass pollen. The individual sera were stored at -20°C. A serum pool was prepared during 1976 from six Manitoba patients highly allergic to grass pollen. The sera were stored at -20°C and have been used in this laboratory for the long-term studies on the allergenic constituents of Kentucky Blue grass pollen.

Radioiodination

The purified *Poa p* I and *Lol p* I* and the murine monoclonal anti-human IgE antibody were labelled with I^{125} by the chloramine T method (61). (This murine anti-human IgE antibody, clone # CIA/E/4.15 was generously supplied by Dr. A.Saxon, UCLA, Los Angeles, CA). Free iodine was removed by passing the reaction mixture through a column of Sephadex G-25 equilibrated with 0.1M phosphate buffer, pH 7.4, containing BSA (1mg/ml).

**Lol p* I was obtained from NIH, Bethesda, MD, USA.

ELISA

ELISA was performed with the 96 well-EIA plate as described in Chapter I. Briefly, the plates were coated with *Lol p* I obtained from NIH (10ug/well) at 4°C overnight, and blocked with 1% BSA in PBS for 2 hrs. Each of Mabs (60,61,62,63,64,290A-167 and 27) diluted with AB was added to the plates (1ug/well) and incubated at 4°C overnight. After washing the plates with PBS containing Tween 20 (1%), a conjugate of rabbit anti-mouse IgG (H+L) and alkaline phosphatase diluted 1000-fold with AB was added and incubated for 1 hr. Following washings, the enzyme substrate, p-nitrophenolphosphate (1mg/ml) was added and the colour reaction was developed for 10 min, then stopped by adding 3M NaOH. The optical density was measured at 410nm using the Microplate Reader.

Competitive inhibition assays

(i) Cross-inhibition of RIA

The relative specificities of the anti-*Poa p* I and the anti-*Lol p* I Mabs were evaluated by a cross inhibition procedure. Solid-phase Mab was prepared by coating the polyvinyl micro-titre plates (Dynatech Lab. Inc., Alexandria, VA) with a Mab (5ug/well) in coating buffer overnight, followed by blocking with 3% BSA in PBS for 3 hrs. Serial dilutions of each purified Mab (triplicate for each dilution) were mixed with either I^{125}-*Poa p* I or I^{125}-*Lol p* I (~$1x10^5$ cpm/well) and incubated on the Mab-solid phase for 4 hrs, at room temp. After washing with PBS, the amount (cpm) of labelled allergen which had bound to the solid-phase was measured with a gamma counter

(Gamma 8000, Beckman Instruments, Inc., Fullerton, CA).

(ii) Cross-inhibition of RAST

A). Micro-titre plates were coated with murine monoclonal anti-human IgE antibody (0.15ug/well) overnight and blocked with 3% BSA in PBS for 3 hrs. (This anti-human IgE antibody, clone # 193.23.C.4.3/56.09, was produced in our Hybridoma Laboratory.) Allergic human serum (diluted 1:4 in assay buffer; 200ul/well) was added to the wells and kept overnight. After washing with PBS, serial dilutions of each Mab (triplicate for each dilution) were mixed with either I^{125}-*Poa p* I or I^{125}-*Lol p* I (~$1x10^5$ cpm/well) and added to the wells for a period of 4 hrs. After washing with PBS again, the amount (cpm) of labelled allergen which had bound to the solid-phase was measured with the gamma counter.

B). Alternately, the micro-titre plates were coated with affinity purified *Poa p* I (2ug/well) overnight and blocked with 3% BSA in PBS for 3 hrs. A pool of six sera obtained from grass allergic patients was diluted 2.5-fold with IB (1% BSA in PBS) and mixed with an equal volume of Mab 290A-167 in varying dilutions. As a control, Mab 27 which is specific to a KBG pollen antigen unrelated to *Poa p* I, was diluted and used in the same way as Mab 290-A-167. One hundred ul of the mixtures was added to each well (triplicate for each dilution). The plates were incubated at 4°C overnight. The extent to which the IgE antibodies bound to the solid-phase *Poa p* I was evaluated by the addition of ^{125}I-mouse monoclonal anti-human IgE antibody (~$1x10^5$ cpm/well).

The degree to which the presence of a Mab in solution inhibited the

binding of the I^{125}-*Poa p* I or I^{125}-*Lol p* I to the solid-phase Mabs or human IgE antibodies or to which the presence of Mab 290A-167 in solution inhibited the bindng of human IgE antibodies to the solid-phase *Poa p* I was assessed in terms of the corresponding decrease in the uptake of the labelled allergen or labelled anti-human IgE antibody onto the wells. This was determined by the formula:

% inhibition=[(A-B)/A]x100;

A: average cpm of the triplicate for each sample in the absence of inhibitor.

B: average cpm of the triplicate for each sample in the presence of inhibitor.

The figures (Fig.7,8,10 and 11) used to illustrate the results of these inhibition assays were prepared with the IBM personal computer and color plotter using the SigmaPlot program.

RESULTS

Antigenic sites on *Poa p* I

The cross-inhibition procedure was employed to compare the relative specificities of the Mabs and thereby to assess the repertoire of epitopes present on *Poa p* I. Thus, the binding of *Poa p* I to solid-phase Mabs 60 or 61 could be completely inhibited in the presence of either Mabs 60 or 61 and Mab 61 was the more efficient inhibitor of the two (Fig.7A,B). These results indicated that Mabs 60 and 61 possessed similar, if not identical, specificities, which has been designated as E_1. By comparison, Mabs 62, 63 and 64 only partially inhibited (60-75%) the binding of *Poa p* I to the solid-phase Mabs 60 or 61. It was not possible to establish whether or not Mabs 62,63 and 64 differed from one another in their relative specificity for *Poa p* I because in the solid-phase form these Mabs lost their antigen-binding property. Nevertheless, on the basis of their similar inhibition profiles shown on Fig.7A,B, it appears that Mabs 62,63 and 64 possess a specificity similar to one another. However, this specificity is not identical to that identified for Mabs 60 or 61, and is designated as E_2.

It was also demonstrated that inhibition of the binding of *Poa p* I to solid-phase Mabs 290A-167 or 348A-6 was obtained only in the presence of the respective homologous Mab (Fig.7C,D). Moreover, since Mab 290A-167 was only poorly able to inhibit (30-40%) the binding of *Poa p* I to the solid-phase Mabs 60 and 61 and Mab 348A-6 had an inhibitory capacity that was not much greater than that obtained with the control Mab 27 (Fig.7A,B), it was

Fig.7. Analyses of antigenic epitopes on *Poa p* I. Serial dilutions of homologous or heterologous Mabs were mixed with I^{125}-*Poa p* I (~$1x10^5$ cpm/well) and added to the plates which were coated with each of the Mabs (Mabs 60,61,290A-167 and 348A-6), respectively. Solid phase Mabs: A, Mab 60; B, Mab 61; C, Mab 290A-167; D, Mab 348A-6.

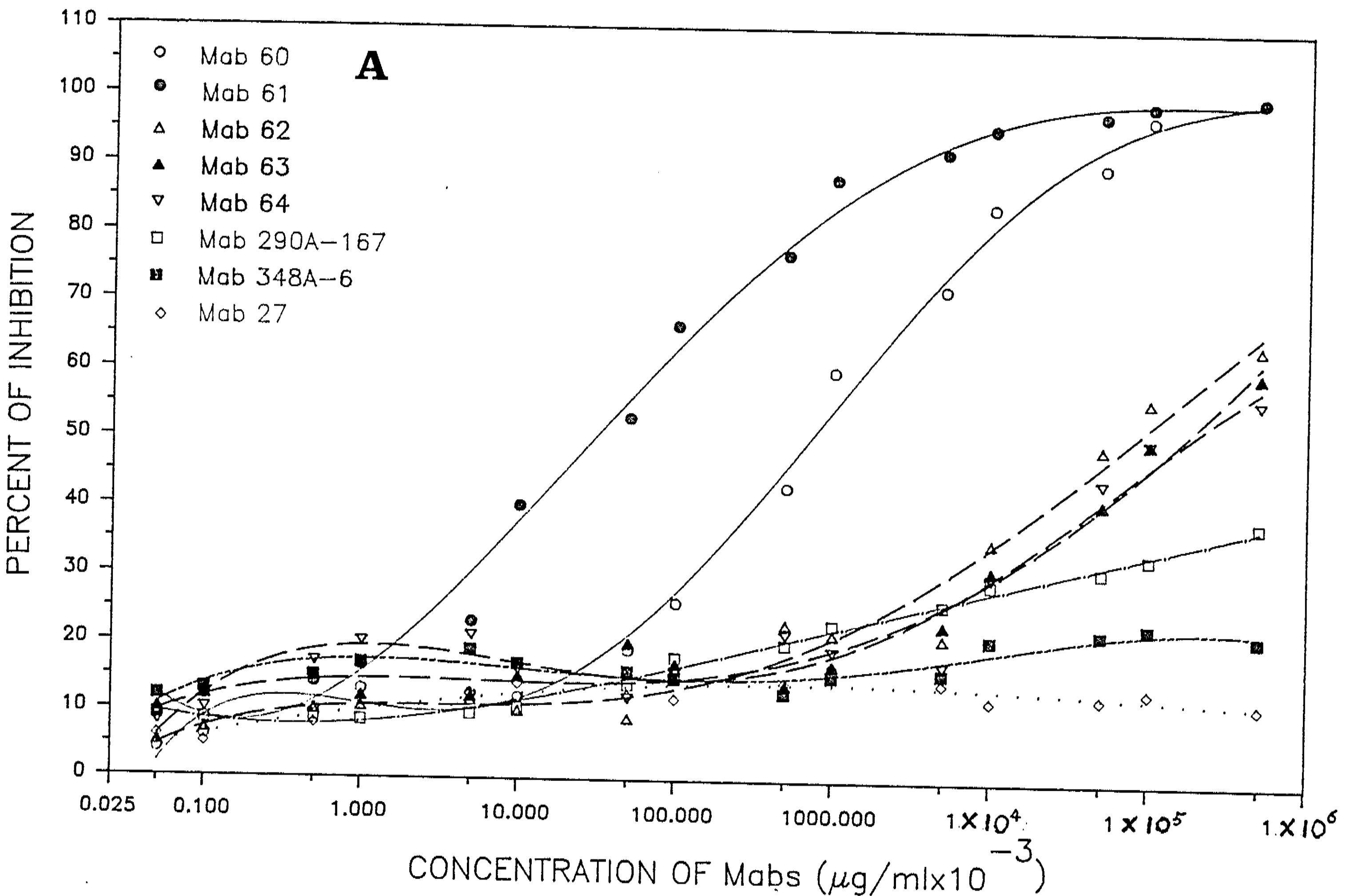

Fig. 7A

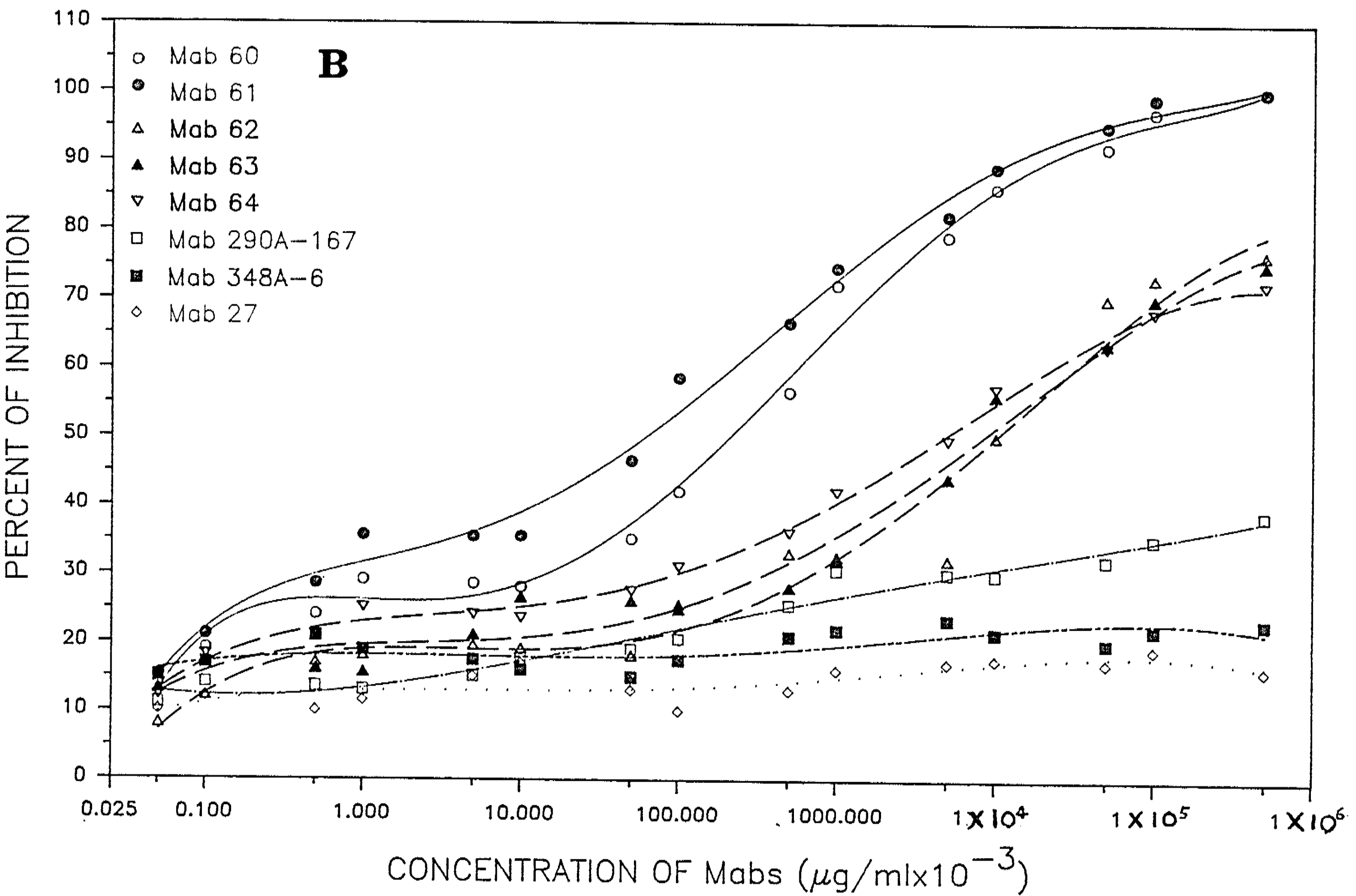

Fig. 7B

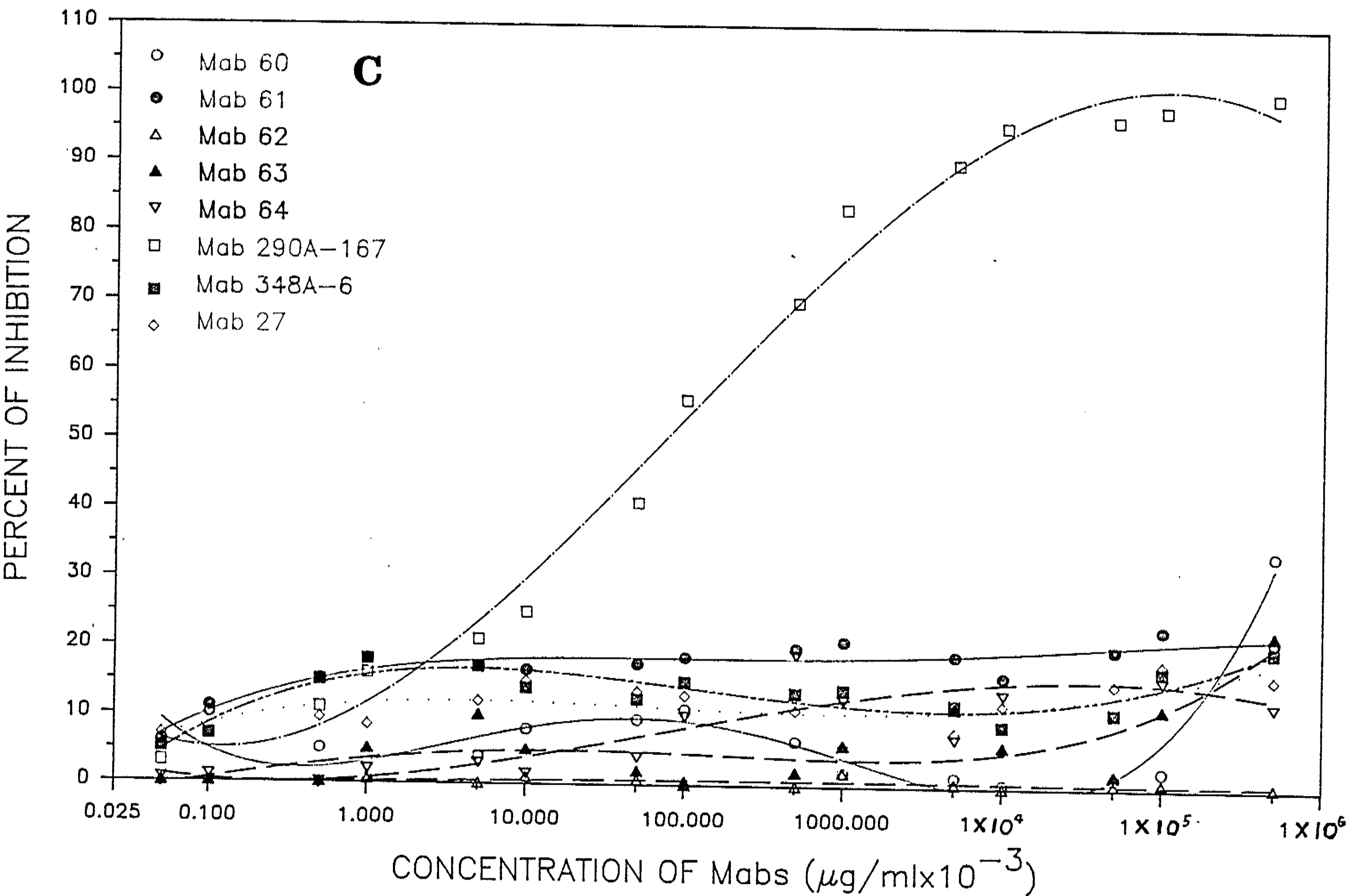

Fig. 7C

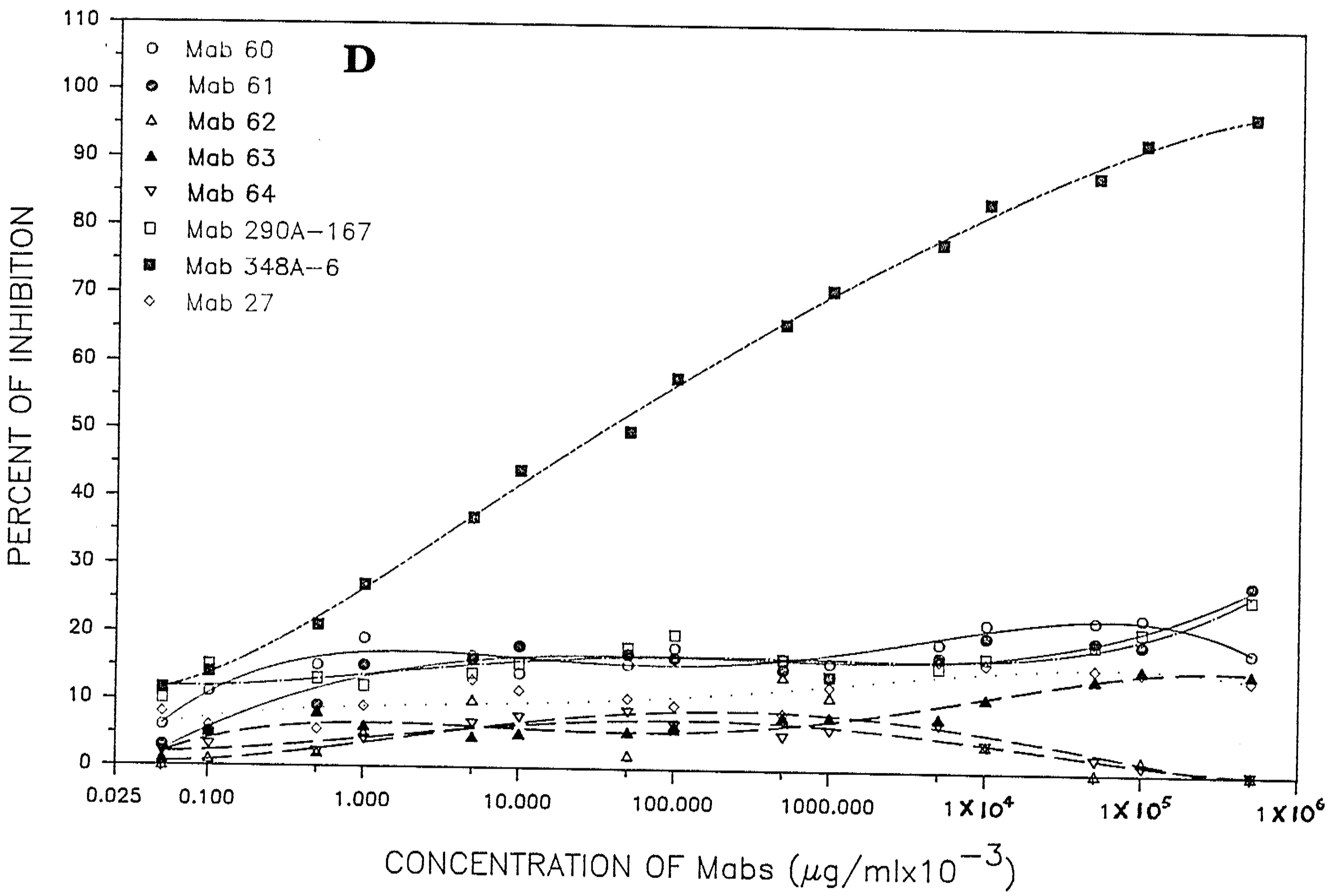

Fig. 7.D

concluded that the specificities of Mabs 290A-167 and 348A-6 were different from one another and also different from those of Mabs 60 and 61 as well as Mabs 62,63 and 64. The specificities of Mabs 290A-167 and 348A-6 were, therefore, respectively designated as E_3 and E_4. As shown in Fig.7C, the inability of Mabs 60 and 61 to inhibit the binding of *Poa p* I to solid-phase Mab 290A-167 may be interpreted that the binding affinity of Mab 290A-167 to E_3 is higher than that of Mabs 60 and 61 to E_1.

In all experiments, Mab 27 was used in the soluble form as a control. Since this Mab is specific for Ag 27 of Kentucky Blue grass pollen (51) and is unable to bind *Poa p* I (60), inhibitions of the order of 15-20% were considered to be non-specific.

From a collective evaluation of the results of the cross-inhibition experiments, it was determined that there are at least 4 epitopes (designated as E_1,E_2,E_3 and E_4) on *Poa p* I, which were recognized by i) Mabs 60 and 61; ii) Mabs 62,63 and 64; iii) Mab 290A-167 and iv) Mab 348A-6, respectively.

Allergenic sites on *Poa p* I

The extent to which the Mabs were able to inhibit the binding of I^{125}-*Poa p* I to solid-phase human IgE antibodies provided an indication of the degree to which the Mabs recognized allergenic epitopes. Clearly, Mab 290A-167 was best able to inhibit the binding of *Poa p* I to the human IgE antibodies of sera BW and JY (Fig.8). By comparison, a lower degree of inhibition (~25%) was obtained in this assay with serum DB. Thus, of the reper-

Fig.8. Analyses of allergenic epitopes on *Poa p* I. Allergic individual serum was added to the plates which were coated with mouse monoclonal anti-human IgE antibody (0.15ug/well). Each of the Mabs (100ug/ml) was mixed with I^{125}-*Poa p* I and added to the plates (~$1x10^5$ cpm/well).

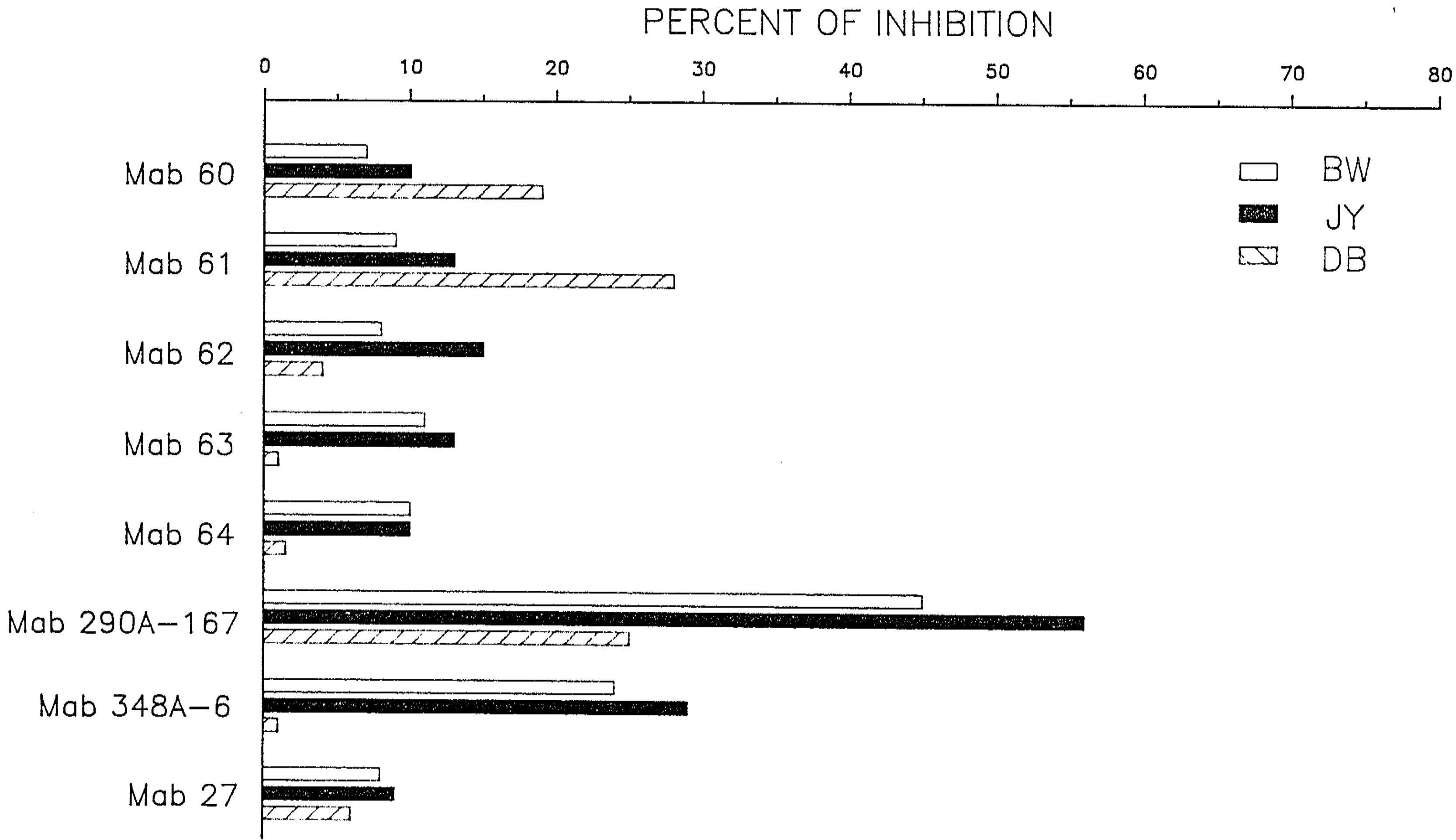

Fig. **8**

toire of specificities of IgE antibodies which bind to *Poa p* I, serum DB appears to contain fewer antibodies which recognize the epitope detected with Mab 290A-167. A similar inhibition profile was obtained in the cases of Mabs 62,63 and 64 with the three sera from allergic individuals (Fig.8). On the other hand, the inhibitions obtained with Mabs 60 and 61 were greater with serum DB compared to that obtained with sera BW and JY. The results of these experiments readily demonstrated differences between the allergic individuals in their levels of IgE antibodies for the four epitopes identified in this study.

Moreover, by using the allergic human sera pool which was obtained from six other patients highly allergic to grass pollen, it was also found that Mab 290A-167 was able to inhibit the binding of human IgE antibodies from this sera pool to *Poa p* I up to the extent of 70% (Fig.9).

From the above observations, it indicated that epitope E_3 recognized by Mab 290A-167 may also constitute an allergenic epitope on *Poa p* I.

Antigenic sites on *Lol p* I

Since the anti-*Poa p* I Mabs 60,61,62,63 and 64 were found by direct binding assays also to recognize the Rye grass pollen allergen *Lol p* I (Table 2), their relative specificities and those of the anti-*Lol p* I Mabs 290A-167 and 348A-6 for binding to *Lol p* I were evaluated by the cross-inhibition procedure. These studies revealed that Mabs 60 and 61 could inhibit virtually completely the binding of *Lol p* I to solid-phase Mab 60 (Fig.10A) or Mab 61 (Fig.10B). In the same manner, it was found that the

Fig.9 <u>Competitive inhibition of RAST</u>. The plates were coated with *Poa p* I (2ug/well). One hundred ul of the mixtures containing 50ul of human allergic serum (diluted 2.5-fold with IB) and 50ul of Mab 290A-167 in varying amounts, were added to each well. Similarly, Mab 27 in varying amounts was used for control. The extent of binding of human IgE to *Poa p* I was evaluated by the addition of ^{125}I-labelled mouse monoclonal anti-human IgE antibody (1x10^{5} cpm/well).

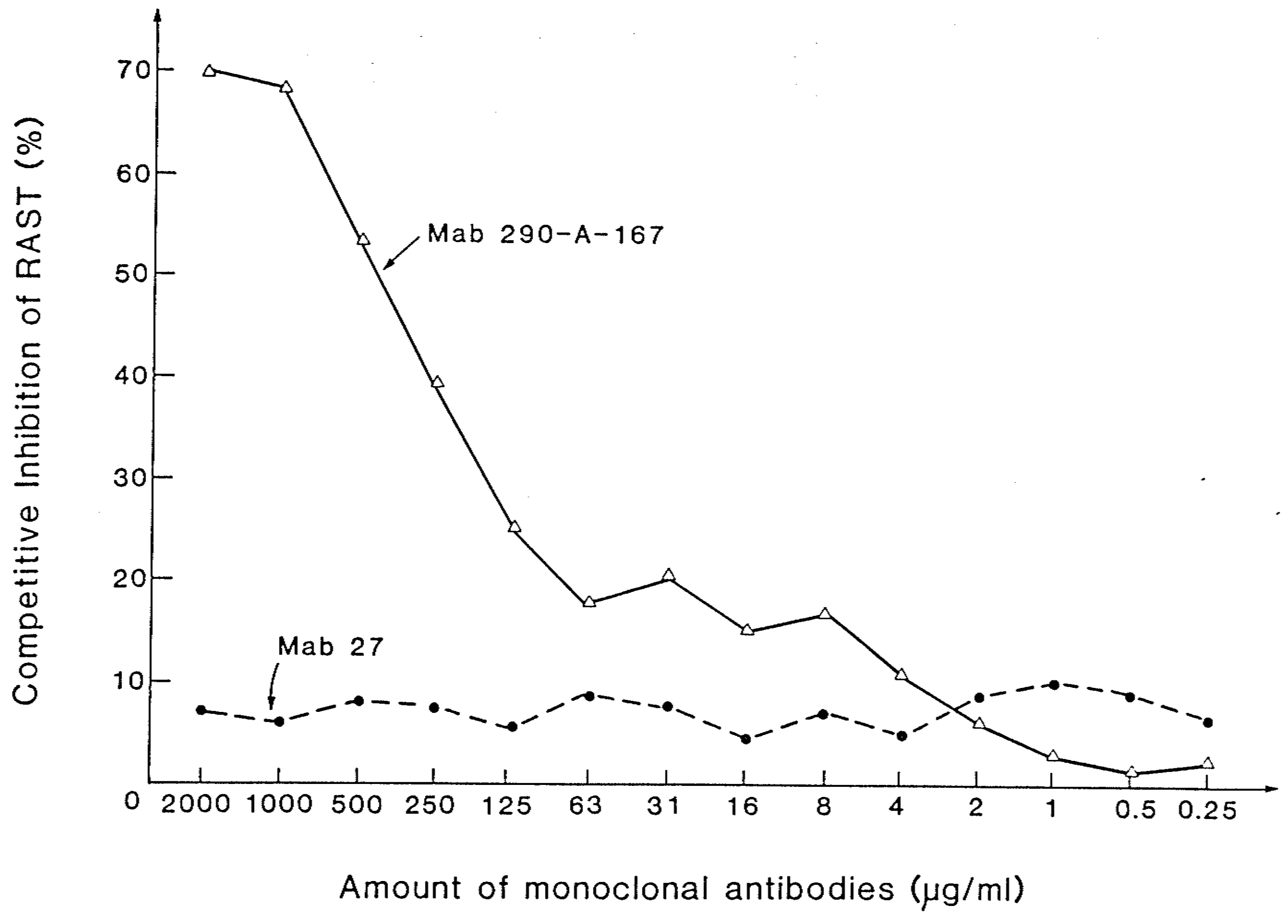

Fig.9

Direct binding of Mabs to *Lol p* I as measured by ELISA*

	Mabs						
	60	61	62	63	64	290A-167	27
O.D $_{410nm}$	0.657	0.700	0.390	0.282	0.345	0.451	0.001

* The plates were coated with *Lol p* I (10ug/well). Each of the Mabs diluted in AB was added to the antigen-coated wells (1ug/well). Rabbit anti-mouse IgG (H+L) alkaline phosphatase conjugate was used as the second antibody. The enzyme-substrate reaction was monitored at 410nm.

Table 2

binding of *Lol p* I to the solid-phase Mabs 60 and 61 was inhibited by the anti-*Lol p* I Mab 290A-167 to the extent of 70%. None of the other Mabs (Mabs 62,63,64 or 348A-6) had a significant inhibitory capacity in this assay (Fig.10A,B).

In the converse situation, i.e. using solid-phase Mab 290A-167, the homologous antibody completely inhibited the binding of *Lol p* I to the solid-phase (Fig.10C). However, Mabs 60 and 61 only partially inhibited (~30%) the binding of *Lol p* I to the solid-phase (Fig.10C). With Mab 348A-6 as the solid-phase, only the homologous antibody could inhibit the binding of *Lol p* I to the solid-phase (Fig.10D).

On the basis of the results of the direct binding assays as well as cross-inhibition experiments, it was concluded that epitopes analogous to those previously designated as E_1,E_2,E_3 and E_4 were also present on *Lol p* I. These experiments revealed there is considerable antigenic homology between *Poa p* I and *Lol p* I.

Allergenic sites on *Lol p* I.

Competitive inhibition assays were performed to evaluate the ability of the Mabs to inhibit the binding of *Lol p* I to human IgE antibodies. Of all the Mabs examined, it was again found that Mab 290A-167 had the greatest capacity to inhibit the binding of *Lol p* I to human IgE antibodies of sera BW and JY (Fig.11). These observations indicated that epitope E_3 also constitutes an allergenic site on *Lol p* I.

Although *Lol p* I was capable of binding to the human IgE antibodies of

Fig.10. Analyses of antigenic epitopes on *Lol p* I. Serial dilutions of homologous or heterologous Mabs were mixed with I^{125}-*Lol p* I (~$1x10^5$ cpm/well) and added to the plates which were coated with each of the Mabs (Mabs 60,61,290A-167 and 348A-6), respectively. Solid phase Mabs: A, Mab 60; B, Mab 61; C, Mab 290A-167; D, Mab 348A-6.

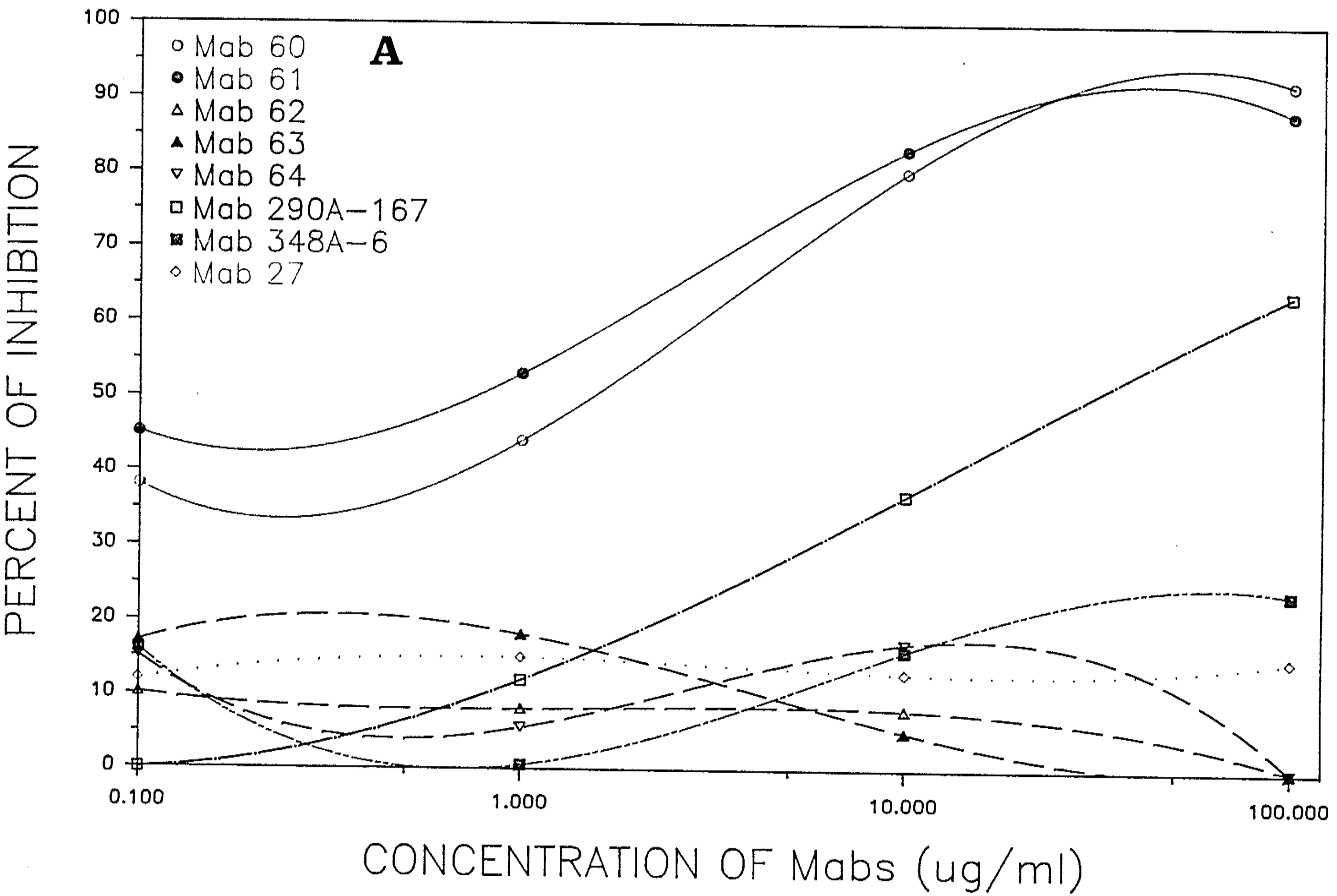

Fig. 10A

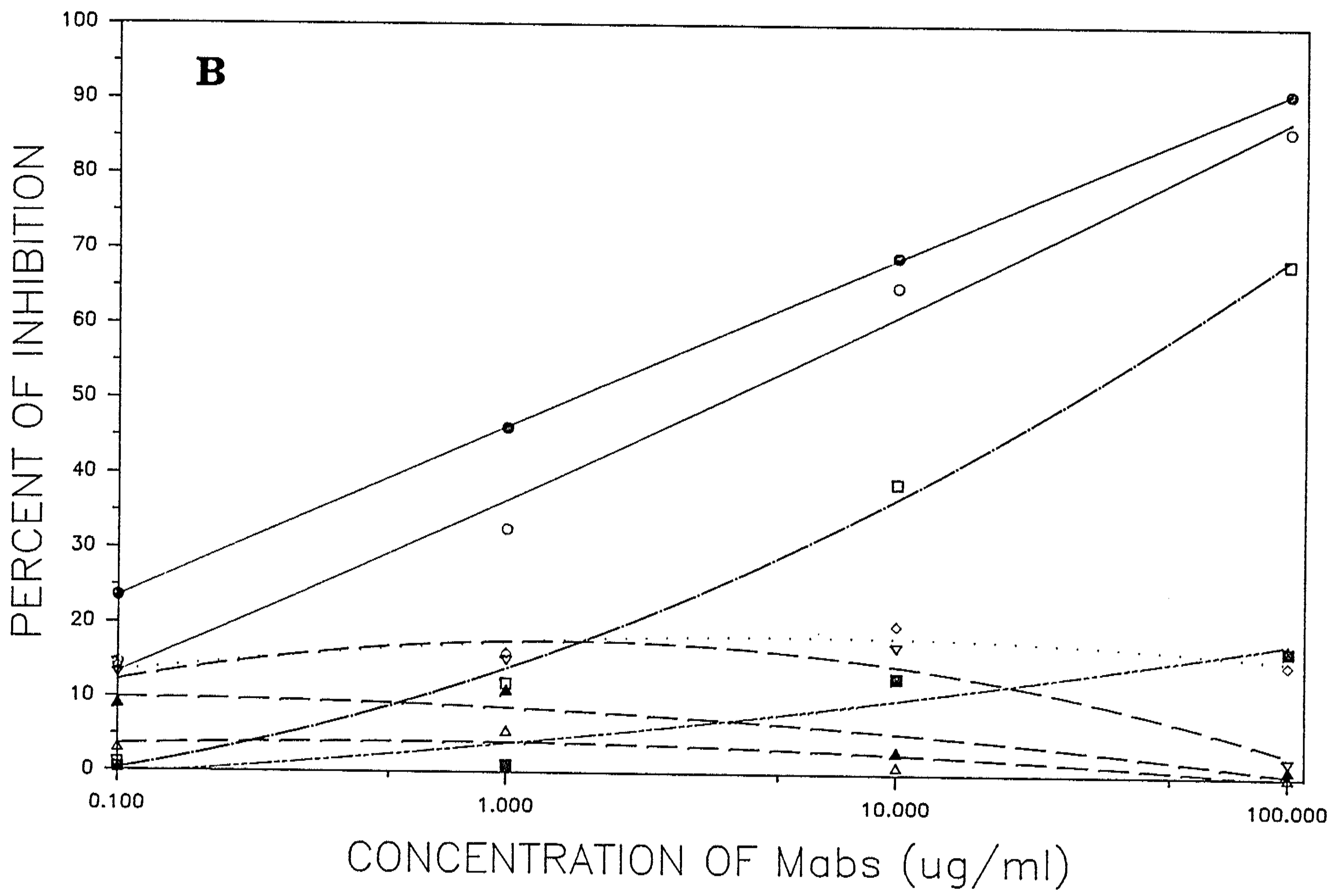

Fig. 10B

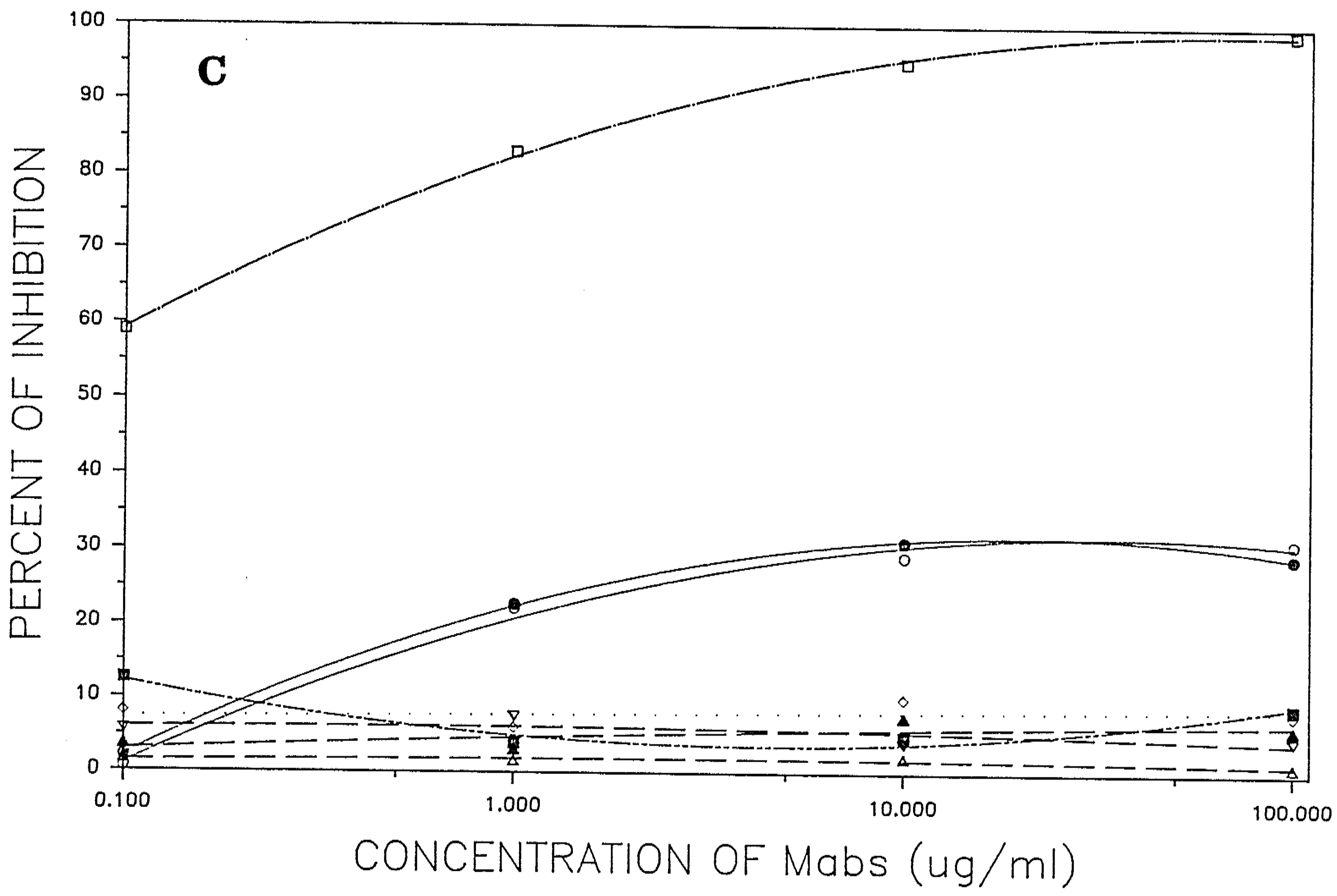

Fig. **10C**

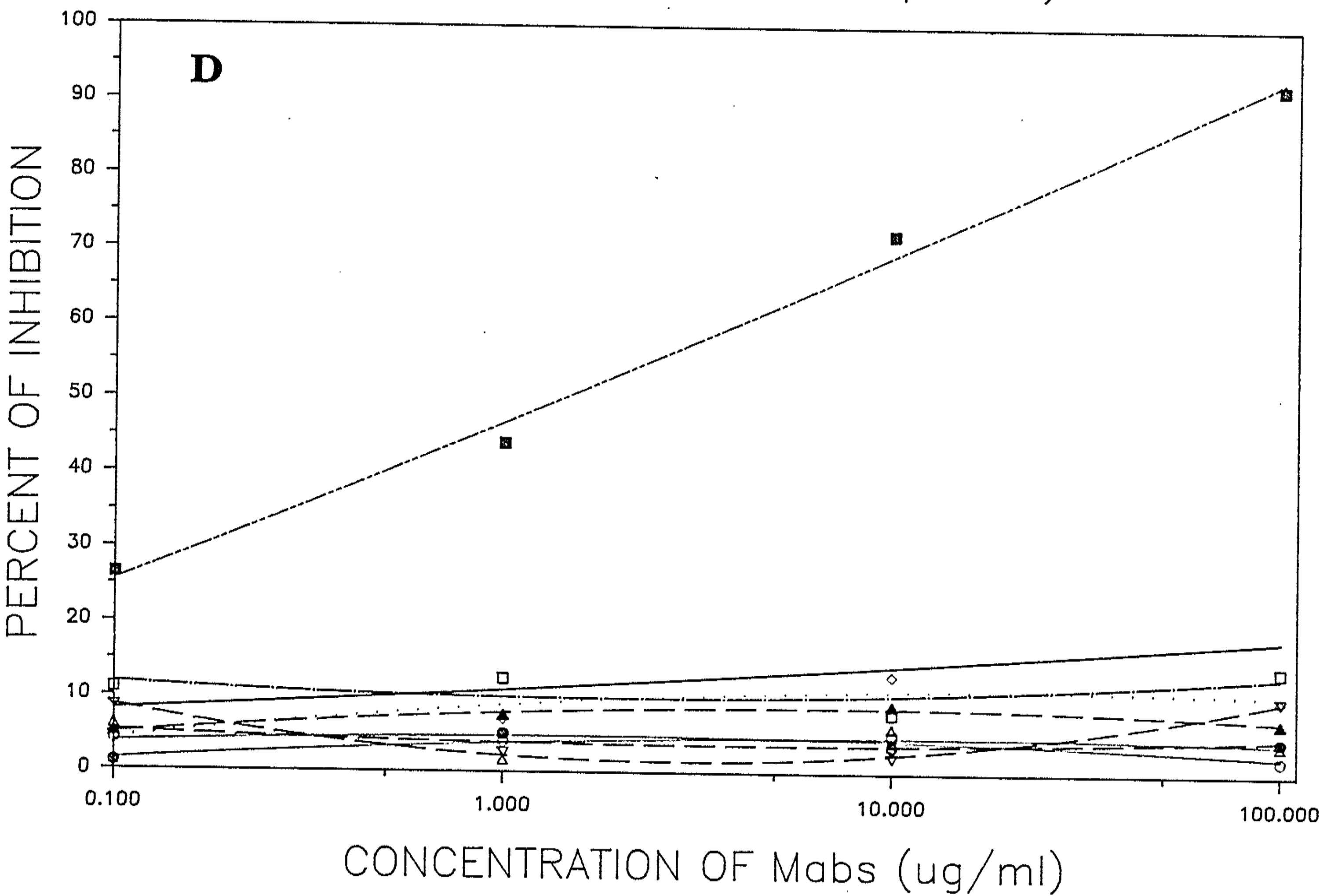

Fig. **10** D

Fig.11. Analyses of allergenic epitopes on *Lol p* I. Allergic individual serum was added to the plates which were coated with mouse monoclonal anti-human IgE antibody (0.15ug/well). Each of the Mabs (100ug/ml) was mixed with I^{125}-*Lol p* I and added to the plates (~1x10^5 cpm/well).

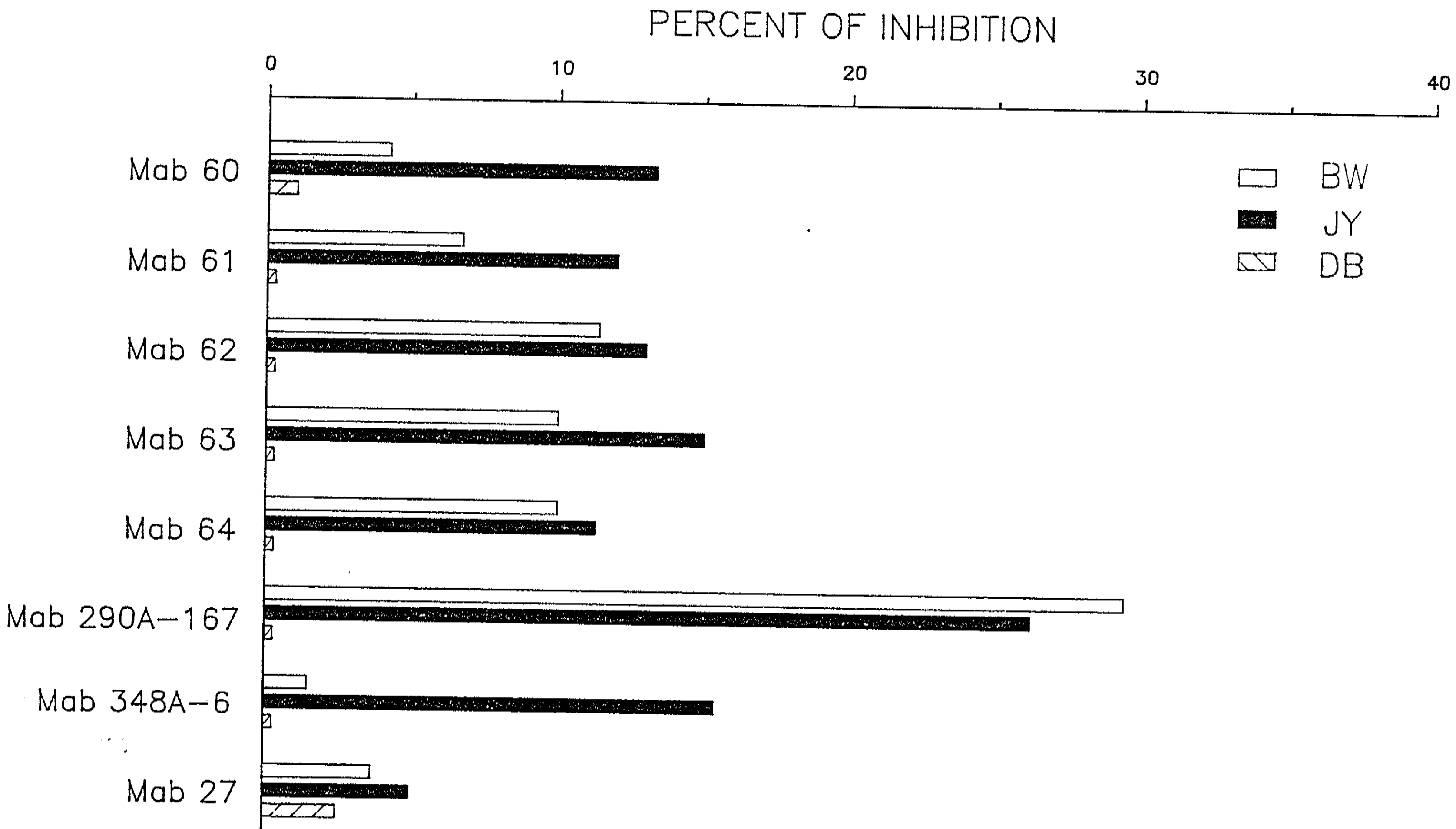

Fig. 11

serum DB (Table 3), none of the Mabs used in the cross-inhibition assays was able to inhibit this binding. On the basis of these observations, it was concluded that the IgE antibodies in human serum DB recognized epitopes on *Lol p* I which were different from those designated as E_1,E_2,E_3 and E_4.

Direct binding of ^{125}I-*Lol p* I to human IgE antibodies*

	IgE Abs from human serum		
	BW	DB	JY
^{125}I-*Lol p* I (cpm)	5887±105	5765±76	3533±132

* The plates were coated with murine monoclonal anti-human IgE antibody (0.15ug/well). Allergic human serum (BW, DB and JY) was individually diluted in IB (1:4) and added to each well. The extent of human IgE binding to *Lol p* I was evaluated by the addition of ^{125}I-*Lol p* I to the wells ($1x10^5$ cpm/well).

Table 3

DISCUSSION

By employing cross-inhibition assays to evaluate and compare the relative specificities of anti-*Poa p* I and anti-*Lol p* I Mabs for both *Poa p* I and *Lol p* I, our present studies identified at least 4 distinct specificities on either allergen. On this basis, it was established that *Poa p* I and *Lol p* I shared extensive antigenic homologies, i.e. they possessed epitopes designated as E_1,E_2,E_3 and E_4. Moreover, the studies further revealed that *Poa p* Ia carried all 4 epitopes whereas *Poa p* Ib only possessed epitopes E_1 and E_3 (Fig.3).

From a consideration of the extent of the inhibitions obtained in the cross-inhibition experiments, it was possible to obtain an insight into the relative spacial relationships of the epitopes identified in this study, i.e. the higher the degree to which a Mab could inhibit the binding of an allergen to a solid-phase Mab, the closer, if not identical, were the two respective epitopes. On this basis, a schematic illustration (Fig.12) has been constructed to indicate the relative proximities of the antigenic and allergenic epitopes according to the collective results of the cross-inhibition studies. This model does not imply a linear relationship of the epitopes along the polypeptide, rather, it is the arrangement for the relative accessibility of the various epitopes to the binding by the Mabs and the human IgE antibodies. Thus, the observation that Mabs 62,63 and 64 were not able to inhibit the binding of Mabs 60 and 61 to *Lol p* I (Fig.10A,B) was interpreted to mean that E_1 and E_2 on *Lol p* I are sufficiently apart from one another, so that the binding of E_1 to Mabs 60 and 61

Fig.12. Schematic illustration of relationships of the epitopes on *Poa p* I and *Lol p* I.

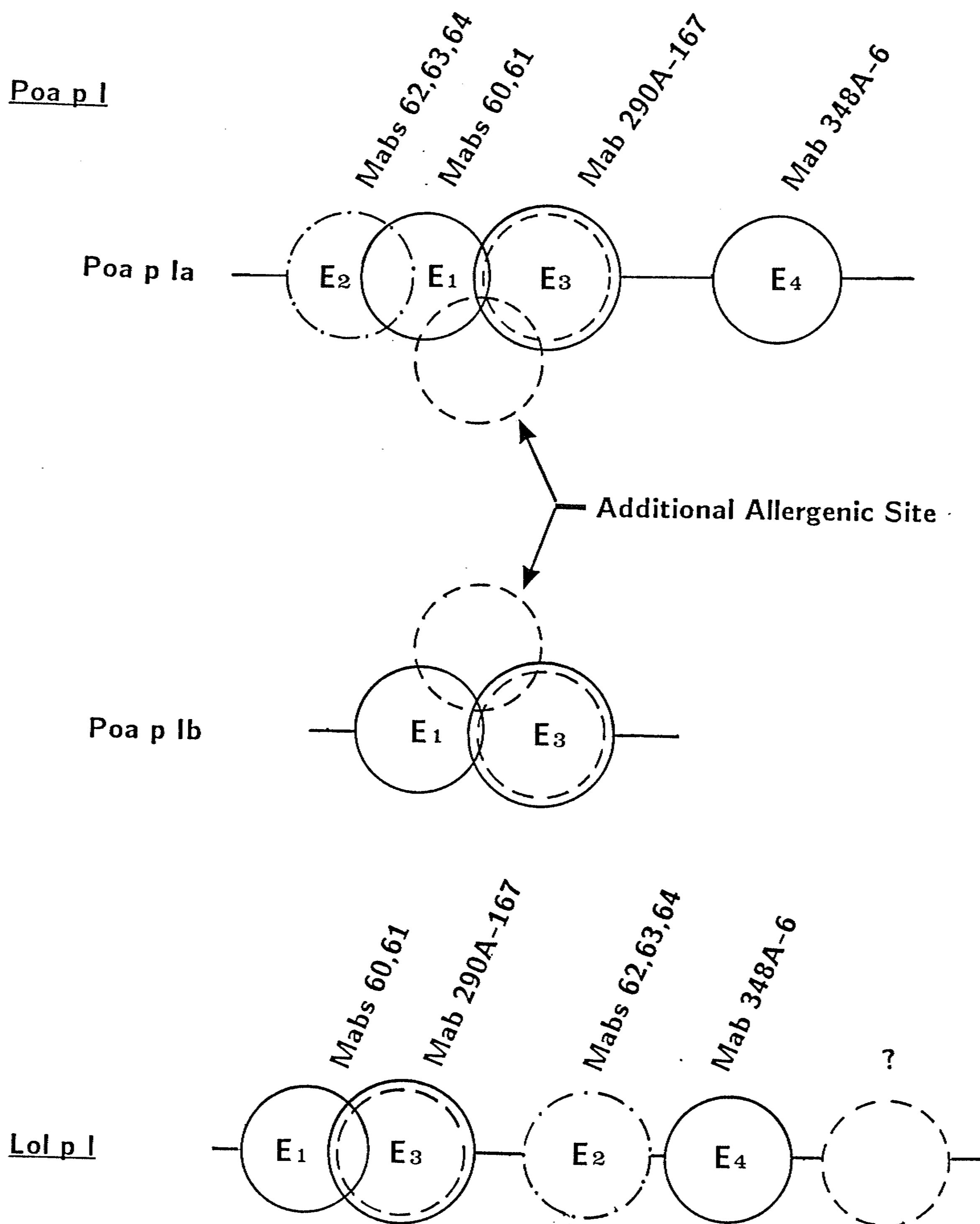

Fig.12

does not interfere with the binding of E_2 to Mabs 62,63 and 64. However, with *Poa p* I, Mabs 62,63 and 64 could partially inhibit its binding to Mabs 60 and 61 (Fig.7A,B). This indicated that E_1 and E_2 on *Poa p* I are relatively close to one another. In the same manner, it may also be suggested that E_1 and E_3 are more closely spaced to each other on *Lol p* I than they are on *Poa p* I. This was based on the observation that Mab 290A-167 inhibited to a greater extent (~70%) the binding of Mabs 60 and 61 to *Lol p* I (Fig.10A,B) than the binding of these Mabs to *Poa p* I (~35%) (Fig.7A,B).

The finding that Mab 290A-167 which recognized epitope E_3 was best able to inhibit the binding of either *Poa p* I or *Lol p* I to the human IgE antibodies from sera BW and JY (Fig.8,11) indicated that epitope E_3 encompasses an allergenic site on both *Poa p* I and *Lol p* I. The observations that Mab 290A-167 also inhibited the binding of *Poa p* I to the human IgE antibodies in the sera pool from six other allergic individuals to an extent of 70% (Fig.9) support the conclusion that E_3 constitutes an allergenic site on *Poa p* I.

The observations that Mabs 290A-167, 60 and 61 could inhibit (20-30%) the binding of *Poa p* I to IgE antibodies of serum DB (Fig.8), whereas these Mabs were unable to inhibit the binding of *Lol p* I to the IgE antibodies of the same serum DB (Fig.11), suggested the presence of an additional allergenic site on *Poa p* I which is close to epitopes E_1 and E_3. The simplest conclusion which can be made for *Lol p* I is that this allergen lacks this additional allergenic determinant found on *Poa p* I. Alternately, if it is suggested that *Lol p* I also possesses this additional allergenic site, it

must be distant from E_1 and E_3 to account for the lack of inhibition of the binding of IgE antibodies by Mabs 60, 61 and 290A-167. Such observations illustrate the difficulties which are encountered when attempts are made to define the fine allergenic specificities and further serve to emphasize the advantage that monoclonal antibodies have over polyclonal IgE antibodies. Nevertheless, the polyclonal human IgE antibodies represent the repertoire of allergenic specificities which are recognized by allergic individuals.

Chapter III

General Discussion

Identification of the repertoire of allergenic epitopes recognized by an allergic individual may facilitate a more accurate diagnosis of the extent of the sensitization to the offending allergen(s). It is then envisaged that treatments be made using tolerogenic derivatives of peptides which encompass those region(s) of the allergenic molecule recognized by the allergic patient. In view of the extensive antigenic and allergenic cross-reactivities among different grass pollens, it is conceivable that treatment with the relevant allergenic constituents of any one pollen may also be efficacious in ameliorating the allergies to the cross-reactive allergens of other grass pollens.

The monoclonal antibodies have proven to be useful not only as site-specific probes but also for investigating the cross-reactivity of antigens from different pollens. A report by Kahn and Marsh (45) showed that of 13 Mabs specific for *Lol p* I, one Mab was able to recognize the related group I antigens found in the pollens of 9 other species of grass including Kentucky Blue grass. Also, Esch *et al* (44) have recently reported that a majority of their anti-*Poa p* I Mabs (25 out of 29) in a direct binding assay cross-reacted with group I antigens from 4 other grass pollens, including Rye grass pollen. Our present studies confirmed their findings that *Poa p* I and *Lol p* I are antigenically cross-reactive, and further identified the homologies of antigenic and allergenic epitopes between these two antigens by using Mabs specific for either *Poa p* I or *Lol p* I and human IgE antibodies from grass allergic individuals.

The cross-inhibition procedure which was successfully employed to

establish the relative specificities of Mabs 60,61,290A-167 and 348A-6 could not be used to distinguish the difference, if any, in the specificity of Mabs 62,63 and 64 for *Poa p* I or *Lol p* I because their antigen-binding properties were lost on their adsorption onto the polyvinyl solid-phase. The loss of the antigen-binding property of the antibody in the solid-phase form has been reported by other investigators (62,63). In such cases, it might mean that these Mabs bind to the polyvinyl surface in a manner that sterically blocks their antigen binding sites.

In this study, it was not always possible to resolve the fine specificities of the Mabs. For example, it was assumed that both Mabs 60 and 61 have the same specificity. This assumption was based on the observations that these Mabs could inhibit virtually completely the binding of *Poa p* I or *Lol p* I to solid-phase Mab 60 (Fig.7A,10A) or Mab 61 (Fig.7B,10B) and they were same in isotype (IgG_1,*k*). However, one can not rule out the possibility that the Mabs may recognize different epitopes which are in close proximity to one another. If this was indeed the situation, the results of the cross-inhibition could not be distinguished from one in which the Mabs had the identical or similar specificity for one epitope.

According to the physical and immunochemical properties of *Lol p* I (group I) antigens, *Poa p* Ia belongs to the group I antigens. *Poa p* Ib has also been categorized as a group I antigen. However, this classification may not be entirely appropriate since *Poa p* Ib's pI (highly basic) is markedly different from that of the group I antigens (acidic). The use of the nomenclature *Poa p* Ia and *Poa p* Ib is provisional and is solely for convenience

to distinguish the acidic component, *Poa p* Ia, from the basic component, *Poa p* Ib. Clearly, further studies are required to elucidate the nature and the extent of the relationship of these two components. Studies comparing related proteins obtained from a variety of sources suggest that the degree of cross-reactivity is directly related to the homology of their amino acid sequences (47). Therefore, establishing a more precise structural homology between *Poa p* I and *Lol p* I must await resolution of amino acid sequences of these allergens.

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