

Identification of two distinct allergenic sites on ryegrass-pollen allergen, *Lol p IV*

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Lol p IV is an important allergen of ryegrass pollen. For the immunochemical identification of antigenic and/or allergenic site(s), murine monoclonal antibodies (MAbs) were prepared against Lol p IV. The hybridoma cell-culture supernatants were screened for anti-Lol p IV antibodies by a combination of ELISA and Western immunoblot analyses. The MAbs were finally purified from ascites on a Mono Q ion-exchange column. In a competitive radioimmunoassay with Lol p IV as the solid phase and ¹²⁵I-labeled MAbs, it was established that MAbs 90, 91, 92, 93, and 94, although they differed in their relative affinities, recognized in common with one another an epitope designated as antigenic site A, whereas MAb 12 recognized a different epitope referred to as site B. Sites A and B were also demonstrated to constitute allergenic determinants of Lol p IV. Differences in the repertoire of specificities of the human IgE antibodies directed to Lol p IV were also demonstrated. Interestingly, it was found that sera from both allergic as well as from nonatopic individuals had IgG antibodies to sites A and/or B. (J ALLERGY CLIN IMMUNOL 1989;83:845-52.)

Pollen extracts are complex mixtures consisting of both allergenic and nonallergenic components. Ryegrass pollen is one of the potent sources of allergens that are responsible for eliciting allergic reactions in grass-sensitive patients. Its various allergenic components have been well defined and characterized.¹⁻⁵ Our laboratory has previously reported on the isolation, purification, and identification of an allergenic glycoprotein, *Lol p IV* (formerly designated as high-molecular-weight basic allergen; this new nomenclature was adopted according to the recommendations by International Union of Immunological Societies⁶) as one of the important allergenic constituents of ryegrass pollen.³ In the present study, the antigenic and/or allergenic site(s) present on this glycoprotein was examined with (1) murine anti-*Lol p IV* MAbs

Abbreviations used

<i>Lol p</i> :	<i>Lolium perenne</i>
MAB:	Monoclonal antibody
KBG:	Kentucky bluegrass
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
RIA:	Radioimmunoassay
PBS:	Phosphate-buffered saline
BSA:	Bovine serum albumin

(MAb 90, 91, 92, 93, and 94), and (2) MAb 12 that was produced in mice against the retentate fraction of KBG in an earlier study in our laboratory and demonstrated to recognize also an epitope on *Lol p IV*.⁵ These MAbs served to define two distinct antigenic epitopes on *Lol p IV*. Moreover, these epitopes were also found to be recognized by human IgE antibodies present in the serum of grass-allergic patients. The identification, quantitation, and the standardization of pollen extracts should be facilitated by the availability of the MAbs of the relevant specificities.⁷

MATERIAL AND METHODS

Isolation of *Lol p IV*

Lol p IV was isolated from the retentate fraction of ryegrass (*Lolium perenne*) pollen according to the procedure

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described earlier.³ The purity of *Lol p IV* was evaluated by SDS-PAGE on a 12% gel.

Immunization

BALB/c mice (Jackson Laboratory, Bar Harbor, Maine) were immunized subcutaneously with doses of 5 µg of *Lol p IV* emulsified in Freund's complete adjuvant on days 1, 30, and 57. The animals were further boosted with *Lol p IV* (5 µg in saline) injected intraperitoneally on days 80, 123, and 135. The individual serum antibody titers were determined by ELISA, as described below.

Cell fusion and production of monoclonal cells

Spleens were removed from immunized mice 3 days after the last immunization. A single cell suspension was prepared from three spleens and washed with RPMI complete (RPMI 1640, Grand Island Biological Co., Grand Island, N.Y.) culture medium before fusion with NS1 myeloma cells (American-Type Culture Collection, Rockville, Md.) and with polyethylene glycol 1540 solution, according to the procedure described earlier.⁸ After centrifugation, the cell pellet was resuspended in RPMI complete culture medium and aliquoted into 24-well Costar plates (Costar Corp., Cambridge, Mass.). The cultures were maintained by alternate addition or replacement of hypoxanthine aminopterin thymidine medium. Four different fusions were performed, generating a total of 384 separate primary cultures. The culture-fluid supernatants were analyzed for antibody activity by ELISA, as described below. The selected cultures were then expanded and retested for antibody production before subcloning. Monoclonal cell lines were produced by the limiting dilution method. Five monoclonal cell lines producing MAbs, which were demonstrated to specifically bind to *Lol p IV* on both ELISA and Western immunoblot analyses (see below), were first expanded by mass culture in flasks and then transferred intraperitoneally into pristane-primed BALB/c mice to produce the ascites.

ELISA

Screening of hybridomas for anti-Lol p IV antibodies. The primary cultures produced from four different fusions were screened by ELISA for antibodies to *Lol p IV*. For this purpose, flat-bottom microtiter plates (Costar serocluster) were coated with 100 µl of solution of *Lol p IV* (0.2 µg in coating buffer, 0.1 mol/L of carbonate-bicarbonate buffer, pH 9.0) per well at 4°C overnight and then blocked with a solution of gelatin (3%) at room temperature for 3 hours. After wells were washed with PBS-Tween 20 (1%) solution, they were incubated overnight with the hybridoma culture-fluid supernatants. After the wells were washed with PBS-Tween, rabbit antimouse IgG peroxidase (1:100 vol/vol; Kirkegaard and Perry Laboratories, Gaithersburg, Md., diluted in PBS containing 1% BSA) was added to the wells and kept for 1 hour. The wells were washed with PBS-Tween, 100 µl of substrate solution was added, and 4 minutes later the absorbance at 490 nm was measured with an

automated ELISA reader (Dynatech Laboratories Inc., Alexandria, Va.).

Determination of MAbs isotype. The isotype of the MAbs was determined by ELISA with the Mono Ab ID ELISA kit (Zymed Laboratories, San Francisco, Calif.) according to manufacturer's instructions. Briefly, ELISA plates were coated with *Lol p IV*, blocked, and washed as described above. Volumes of 100 µl of the culture-fluid supernatants containing the MAb were added to the wells and incubated overnight. After the plate was washed with the PBS-Tween solution, rabbit antibodies specific for one of the immunoglobulin classes or their subclasses were added to the wells and kept at room temperature for 1 hour. The wells were again washed before incubation with a goat antirabbit immunoglobulin-alkaline phosphatase conjugate for 1 hour. After the plates were washed with the PBS-Tween solution, substrate solution (1 mg/ml of paranitrophenyl phosphate in 10% diethanolamine buffer, pH 9.8) was added, and the reaction was allowed to develop for 40 minutes. The reaction was stopped by addition of 50 µl of 3 mol/L NaOH to each well. The optical density was read at 410 nm with the automated ELISA reader. All the MAbs were found to be of the IgG1 (*kappa*) class.

Determination of the concentration of IgG in monoclonal hybridoma cell culture-fluid supernatants. The concentration of IgG present in the culture-fluid supernatants was determined in terms of the degree that these supernatants could inhibit the binding of antimouse IgG-alkaline phosphatase conjugate to solid-phase MAb 12. For this purpose, the ELISA plates were coated with purified MAb 12 (0.1 µg per well) and blocked with gelatin, as described above. The assay was standardized with purified MAb 12 as the inhibitor. Thus, the rabbit antimouse IgG-alkaline phosphatase (diluted 1:1000 in assay buffer [PBS containing 0.5% BSA], Sigma Chemical Co., St. Louis, Mo.) was mixed with purified MAb 12 at different concentrations and kept at room temperature for 3 hours before addition of the mixtures to the MAb 12-coated wells. One hour later, the wells were washed with PBS-Tween, and the substrate solution was added. After a period of 10 minutes, the absorbance at 410 nm was measured. The extent of the inhibitions were calculated according to the formula: inhibition (%) = $(A - B)/A \times 100$, where A equals absorbance in the absence of inhibitor and B equals absorbance in the presence of inhibitor.

The culture-fluid supernatants were similarly used in place of purified MAb 12, and their immunoglobulin concentration was determined with reference to the standard graph (Fig. 1).

Ranking the relative affinities of the MAbs. The culture-fluid supernatants of clones secreting MAbs 90 to 94 and the purified MAb 12 preparation were first appropriately diluted with assay buffer to equalize the concentrations of IgG. The MAbs were then serially diluted and added to *Lol p IV*-coated microtiter-plate wells, and the ELISA was continued according to the procedure described earlier in this article. The ELISA absorbance values reflected the relative binding affinity of the MAbs for *Lol p IV*, and they

were ranked accordingly with the method described by Heyningen et al.⁹

Titration of anti-Lol p IV IgG antibodies in human sera. The ELISA plates were coated with *Lol p IV* and blocked with gelatin, described earlier in this article. This was followed by adding volumes of 100 μ l of allergic human serum, diluted twofold, to the wells. The next day, the plates were washed with PBS, and rabbit antihuman IgG-alkaline phosphatase (1:1000 vol/vol diluted in assay buffer, 100 μ l per well; Sigma Chemical Co.) was added to the wells and kept for 1 hour. After wells were washed with the PBS-Tween solution, substrate solution (paranitrophenyl phosphate) was added, and absorbance was read after 10 minutes at 410 nm on ELISA reader.

Western immunoblot

Retentate fraction of ryegrass pollen extract or *Lol p IV* was subjected to SDS-PAGE in 12% polyacrylamide gel. After electrophoretic transfer of the components onto nitrocellulose membrane and blocking with a solution of gelatin (3%), the membrane was immersed in a hybridoma cell culture-fluid supernatant to allow for binding of antibodies to *Lol p IV* protein. The immunoblot was developed with the affinity-purified goat antimouse IgG peroxidase conjugate (Bio-Rad Laboratories, Richmond, Calif.) according to the procedure described earlier.⁵

Production of ascitic fluids

Five monoclonal cell lines (256.41, A1.4/44 [MAb 90]; 256.41, A1.4/63 [MAb 91]; 258.41, C3.2/123 [MAb 92]; 258.41, C3.2/164 [MAb 93]; and 258.41, C3.2/4 [MAb 94]) were expanded in vitro and approximately 5×10^6 monoclonal cells were injected into the peritoneal cavity of pristane-primed BALB/c mice. The ascitic fluids were collected 20 to 30 days later by puncturing the abdominal wall with a 20-gauge needle and allowing the ascites to leak out. The MAb 12 (cell line 86.6 B3.5/14-15) was one of the MAbs produced against the retentate fraction of KBG and was demonstrated in a previous study to recognize also an epitope on *Lol p IV*.⁵

Purification of MAbs

The immunoglobulin fractions of MAbs 90 to 94 were obtained from the corresponding ascitic fluids by precipitation with a solution of ammonium sulfate at a final saturation of 50%. After dialysis against 0.02 mol/L of triethanolamine, the immunoglobulin fractions were further purified by fast protein liquid chromatography on an anion exchange Mono Q column (Pharmacia Biotechnology International, Uppsala, Sweden) with 0.02 mol/L of triethanolamine (pH 7.7) as the starting buffer and 0.02 mol/L of triethanolamine containing 0.35 mol/L of NaCl as the limiting buffer. A linear gradient was generated at a flow rate of 1 ml/min during a period of 30 minutes. The eluted protein fractions were monitored for *Lol p IV*-specific IgG antibody activity by ELISA with antimouse IgG alkaline phosphatase, as described above. The fractions containing

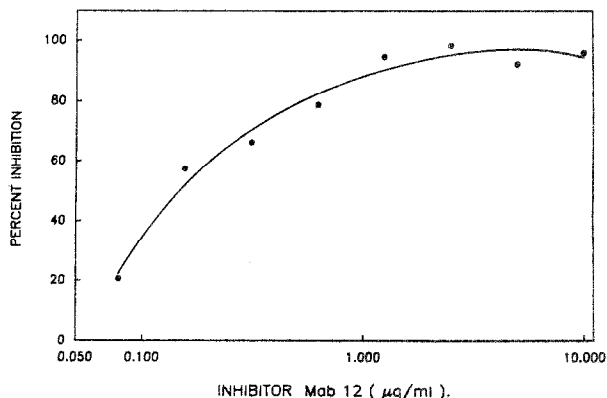


FIG. 1. Standardization of the ELISA-inhibition method for measuring the IgG concentration in MAB culture-fluid supernatants. The standard graph was constructed by plotting the degree of inhibition of binding of antimouse IgG-alkaline phosphatase to MAB 12-coated plates in the presence of known amounts of purified MAB 12.

antibody were pooled and analyzed by SDS-PAGE. MAB 12 was purified by combination of ammonium sulfate precipitation and gel filtration on Ultrogel ACA 44, LKB, Bromma, Sweden, as described elsewhere.¹⁰

RIA

Radioiodination. The purified MAbs or *Lol p IV* were radiolabeled with ^{125} I by the chloramine-T method.¹¹ The unbound iodine was removed by dialysis against PBS. The radiolabeled proteins were 70% to 90% precipitable with 20% trichloroacetic acid.

Identification of antigenic epitopes on *Lol p IV* by competitive RIA with murine MAbs. The flexible polyvinyl microtiter plates (Dynatech Laboratories, Inc.) were coated with *Lol p IV* (0.2 μ g per well) in coating buffer, blocked with 2% BSA in PBS for 3 hours, and incubated overnight with ^{125} I-labeled MAB (approximately 100,000 cpm per well). The wells were subsequently washed with PBS and counted in the gamma counter (Beckman Instruments, Inc., Fullerton, Calif.). For inhibition experiments, dilutions of ascitic fluids containing the MAB were premixed with an ^{125} I-labeled-purified MAB and then incubated overnight on *Lol p IV*-coated plates. The extent to which each of the six ^{125} I-labeled MAbs were inhibited from binding to solid-phase *Lol p IV* in the presence of the different ascites containing MAbs was calculated according to the formula: percent inhibition = $(A - B)/A \times 100$, where A equals counts per minute in the absence of inhibitor, and B equals counts per minute in the presence of inhibitor.

For control, MAbs with specificities unrelated to ryegrass allergen *Lol p IV*, that is, MAB 27 (antigen 27 of KBG¹²) and MAB GL-74 (antiovalbumin, produced in Dr. Sehon's laboratory), were used in the form of ascitic fluid diluted fiftyfold with assay buffer.

Cross-inhibitions between the binding of murine MAbs and human IgE antibodies to *Lol p IV*. The specificities of

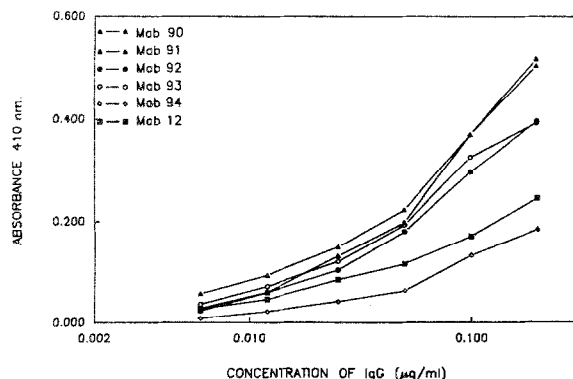


FIG. 2. Relative binding affinity of MAbs to solid-phase *Lol p IV*. All the MAbs were diluted to equal IgG concentrations, and the ELISA were performed with *Lol p IV*-coated plates, as described in Material and Methods.

polyclonal IgE antibodies present in the sera of humans allergic to *Lol p IV* and mouse monoclonal anti-*Lol p IV* antibodies were compared by an RIA-inhibition procedure described earlier.¹³ Briefly, polyvinyl microtiter plates were coated with mouse monoclonal antihuman IgE (cell line 193.23.C.4.3/56.09 produced in our hybridoma laboratory; 0.15 µg per well) blocked with 2% BSA in PBS, followed by overnight incubation with the individual sera of grass-allergic patients. After wells were washed with PBS, ¹²⁵I-labeled *Lol p IV* (approximately 100,000 cpm) was added and incubated overnight. To examine the possibility that some of the allergenic determinants on *Lol p IV* could also be recognized by murine MAbs, the MAbs (ascitic fluids diluted 1:5 in assay buffer) were mixed with ¹²⁵I-labeled *Lol p IV* for 3 hours before their addition to the wells. The extent to which the labeled *Lol p IV* was inhibited from binding to human IgE antibodies was calculated with the formula described earlier in this article.

Cross-inhibitions between the binding of murine MAbs and human serum antibodies to *Lol p IV*. The cross-reactivity of polyclonal human antibodies (predominantly IgG and IgE) and mouse monoclonal anti-*Lol p IV* antibodies was examined in a cross-inhibition RIA. The polyvinyl microtiter plates were coated with *Lol p IV* (0.2 µg per well) and blocked with 2% BSA in PBS. For the cross-inhibition experiments, an ¹²⁵I-labeled preparation of MAb 12 or MAb 90 (approximately 100,000 cpm) was premixed with 100 µl of human serum at room temperature for 3 hours, added to *Lol p IV*-coated wells, and kept overnight at room temperature. For control, 2% BSA was used instead of human serum. The percent inhibition of the binding of any of the ¹²⁵I-labeled MAbs to the *Lol p IV* solid phase was calculated with the formula described earlier in this article.

RESULTS

Production of MAbs

Of six BALB/c mice immunized with *Lol p IV*, four had a high serum antibody titer (>1:16,000) for *Lol p IV*. Spleen cells from the three best responding

animals were used for four different fusions with the NS1 myeloma cell line. The culture-fluid supernatants obtained from 384 primary cultures were screened for anti-*Lol p IV* antibody activity by ELISA. The 156 hybridoma cell clones secreting anti-*Lol p IV* antibodies (ELISA absorbance >0.075) were further expanded in tissue culture and retested by ELISA. Of these cell clones, a total of 42 clones were selected on the basis of their antibody activity (ELISA absorbance >0.15). Their antibody specificity for recognizing *Lol p IV* was reconfirmed by the Western immunoblot method. Five monoclonal cell lines produced by the limiting dilution method were further expanded in vitro and propagated in the peritoneal cavity of mice for the production of the MAbs.

Relative affinities of anti-*Lol p IV* MAbs

The concentration of IgG in the culture-fluid supernatants of monoclonal hybridomas secreting MAbs 90, 91, 92, 93, and 94, as determined by the ELISA-inhibition method (Fig. 1), was found to be 15, 14, 9.5, 13.5, and 10.5 µg/ml, respectively. The ELISA values obtained for the binding of each of these monoclonal antibodies, at equal immunoglobulin concentrations, to solid-phase *Lol p IV* provided an index of their relative affinities (Fig. 2). There was no significant difference in ELISA values for MAb 90, 91, 92, and 93. In contrast, MAb 94 and MAb 12 had comparatively lower ELISA values. Thus, in terms of their relative affinities for *Lol p IV*, these MAbs were ranked in the following order: MAb 90 = MAb 91 = MAb 92 = MAb 93 > MAb 12 > MAb 94.

Identification of antigenic sites on *Lol p IV*

The specificities of the MAbs were compared by a competitive RIA-inhibition test with *Lol p IV* solid phase. In this assay the binding of ¹²⁵I-labeled MAb 90 to *Lol p IV*-coated wells was inhibited by MAb 90, 91, 92, 93, and 94 but not by MAb 12 (Fig. 3, A). Furthermore, it can be observed that MAbs 90 to 93 produced similar inhibition patterns, whereas MAb 94 resulted in much lower degree of inhibition. Similar patterns for the inhibition of the binding of ¹²⁵I-labeled preparations of MAbs 91, 92, or 93 to *Lol p IV* solid phase were obtained by MAbs 90 to 94 (Fig. 3, B, C, and D). These results suggested that MAbs 90 to 93 were recognizing the same or similar epitope, which has been designated as site A. It was interesting to observe that MAb 94 inhibited the binding of ¹²⁵I-labeled MAb 94 to the solid-phase antigen to a lesser degree compared to the inhibitions obtained with MAbs 90 to 93 (Fig. 3, E). It is therefore suggested that the low degree of inhibition observed with MAb 94 was due to its low affinity for site A of *Lol p IV*.

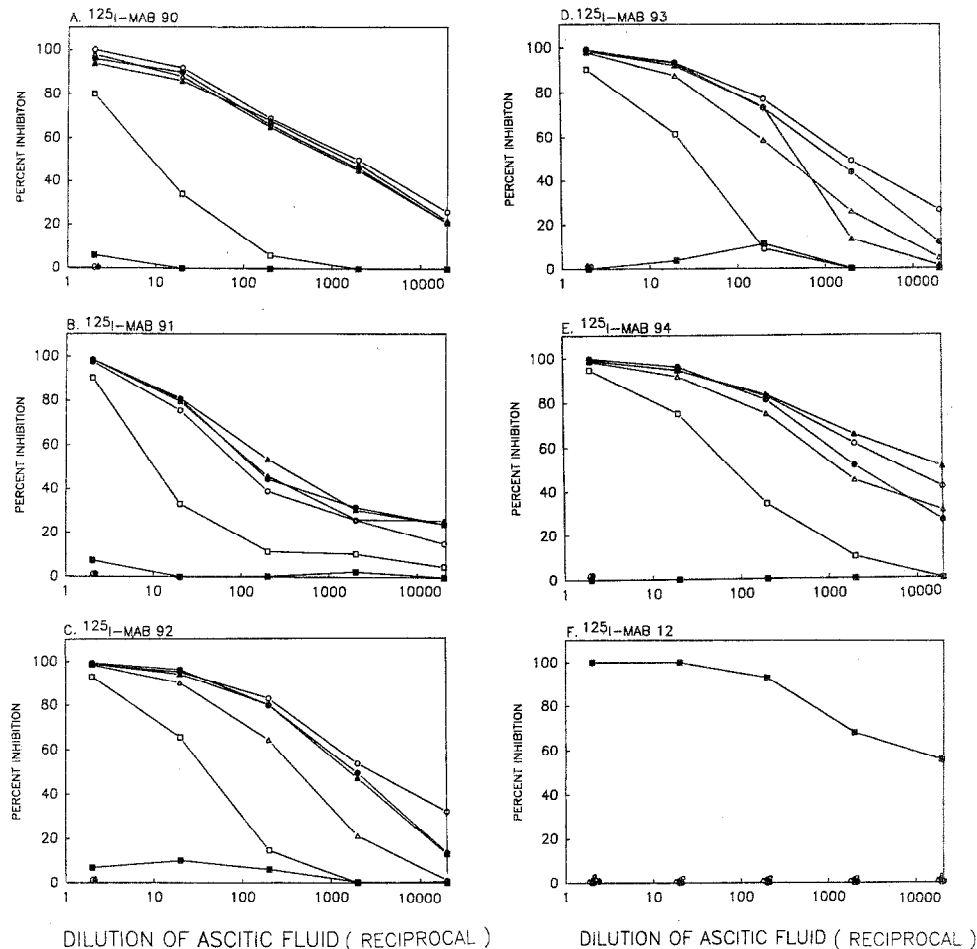


FIG. 3. Comparisons of the specificities of MABs to *Lol p IV* by the RIA cross-inhibition procedure. Each of the ^{125}I -labeled purified MABs was mixed with one of the MAB, in different dilutions, as the inhibitor, before adding to *Lol p IV*-coated microtiter plates. The results indicate the degree of inhibition of the binding of the ^{125}I -labeled MAB preparations (A, MAB 90; B, MAB 91; C, MAB 92; D, MAB 93; E, MAB 94; and F, MAB 12) to solid-phase *Lol p IV* by MAB 90 (●), MAB 91 (○), MAB 92 (▲), MAB 93 (△), MAB 94 (□), and MAB 12 (■). For control, ascitic fluids containing MAB 27 (○) and MAB GL-74 (●) (used only at the lowest dilution) were used in place of inhibitor.

Clearly, MAB 12 identified a site (designated as B) distinct from site A because none of the MABs 90 to 94 was able to inhibit the binding of ^{125}I -labeled MAB 12 to *Lol p IV* (Fig. 3, F). Also, it is evident from Fig. 3, A to E, that MAB 12 was not capable of inhibiting the binding of any of the ^{125}I -labeled MABs 90 to 94 to solid-phase *Lol p IV*. From the collective data of those cross-inhibition experiments, it was concluded that MABs 90 to 94 were recognizing, in common with one another, a site A, whereas MAB 12 was recognizing a distinctly different epitope, designated as site B.

Allergenic site(s) of *Lol p IV* are recognized by murine MABs

The possibility that the sites recognized by the MABs constituted also allergenic site(s) was assessed

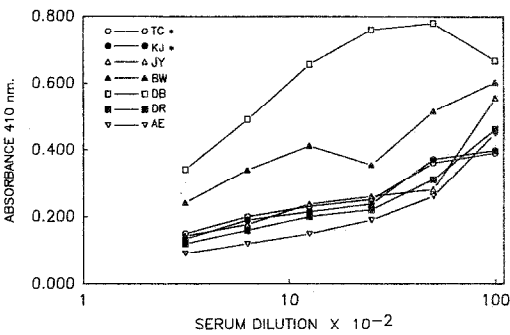


FIG. 4. ELISA for demonstration of anti-*Lol p IV* IgG antibodies in human sera. Twofold serial dilutions of the human sera were added to *Lol p IV*-coated plates. The presence of human IgG antibodies bound to the solid phase was detected by the addition of rabbit antihuman IgG-alkaline phosphatase, as described in Material and Methods.

TABLE I. Inhibition of the binding of human anti-*Lol p IV* IgE antibodies to ¹²⁵I-labeled *Lol p IV* by anti-*Lol p IV* MABs

Patient	Maximum* binding (cpm)	% Inhibition by MAB†						MOPC-21A‡	Normal serum IgG
		90	91	92	93	94	12		
B. W.	1573	20 ± 1§	25 ± 2	19 ± 1	27 ± 1	14 ± 1	30 ± 2	0.6 ± 1	2 ± 1
D. B.	1315	34 ± 2	34 ± 3	25 ± 1	22 ± 1	16 ± 1	17 ± 2	1 ± 1	1 ± 1
J. Y.	1198	40 ± 1	35 ± 1	33 ± 2	26 ± 1	20 ± 1	57 ± 3	0	5 ± 1
D. R.	1100	22 ± 1	25 ± 1	30 ± 1	19 ± 2	15 ± 2	52 ± 2	2 ± 1	3 ± 1
A. E.	1214	0	0	0	0	0	22 ± 1	0	0

Murine monoclonal antihuman IgE was coated onto polyvinyl microtiter plates followed by addition of allergic human serum. ¹²⁵I-labeled *Lol p IV* (approximately 100,000 cpm) was premixed with mouse anti-*Lol p IV* MABs for 3 hours before overnight incubation on the microtiter plates.

*Counts per minute of ¹²⁵I-labeled *Lol p IV* that bound to the human IgE antibodies to *Lol p IV* in absence of a monoclonal antibody.

†Percent inhibition of the binding of ¹²⁵I-labeled *Lol p IV* to the human IgE antibodies by murine monoclonal antibodies to anti-*Lol p IV* MABs (ascites diluted 1:5 in assay buffer).

‡MOPC-21A IgG and normal BALB/c mouse serum IgG (at concentrations of 50 µg/ml) served as controls.

§Standard deviation.

TABLE II. Inhibition of the binding of ¹²⁵I-labeled MABs 90 and 12 to solid-phase *Lol p IV* by anti-*Lol p IV* antibodies present in human sera

Serum	% Inhibition*	
	MAB 90 (anti-site A)	MAB 12 (anti-site B)
Atopic		
B. W.	55 ± 1	36 ± 2
D. B.	46 ± 2	6 ± 3
J. Y.	55 ± 1	0
D. R.	48 ± 1	37 ± 1
A. E.	42 ± 2	29 ± 1
Nonatopic		
K. J.	81 ± 3	45 ± 2
T. C.	0	40 ± 2

*The extent to which the presence of a human serum inhibited the binding of an ¹²⁵I-labeled MAB to solid-phase *Lol p IV* was determined with reference to the maximum binding of the ¹²⁵I-labeled MAB in the absence of the human serum.

in terms of the capacity of the MABs to inhibit the binding between ¹²⁵I-labeled *Lol p IV* and IgE antibodies present in allergic sera. The results presented in Table I revealed that MABs 90 to 94 were capable of inhibiting, from 14% to 40%, the binding between ¹²⁵I-labeled *Lol p IV* and IgE antibodies present in the sera of four allergic patients. None of these MABs was able to inhibit the binding between the IgE antibodies present in the serum of patient A. E. and the ¹²⁵I-labeled *Lol p IV*. By comparison, MAB 12 inhibited from 17% to 57% the binding of *Lol p IV* to

human IgE antibodies present in the five atopic patients' sera examined. These results indicated that MABs 90 to 94 and MAB 12 bound to allergenic sites recognized also by human IgE antibodies.

IgG antibodies to *Lol p IV* in human sera

All the human sera examined in the present study were found to possess IgG antibodies to *Lol p IV* (Fig. 4). Moreover, two nonatopic human sera (T. C. and K. J.) that were found by RAST (results not presented) to be devoid of anti-*Lol p IV* IgE antibodies were, nevertheless, found to possess IgG antibodies to *Lol p IV*.

Murine MABs and antibodies present in human sera may recognize the same determinant(s) on *Lol p IV*

All sera, except from patient T. C., significantly inhibited the binding of ¹²⁵I-labeled MAB 90 to *Lol p IV* (Table II). Similarly, with the exception of sera from patients D. B. and J. Y., all sera could inhibit the binding of ¹²⁵I-labeled MAB 12 to *Lol p IV*. Collectively, these results revealed that sera obtained from ryegrass-allergic individuals and nonatopic individuals could also possess non-IgE antibodies to either site A and/or site B on *Lol p IV*.

DISCUSSION

Lol p IV, a high-molecular-weight (56,800 daltons) basic protein is one of the important allergens of ryegrass pollen.³ With MABs (MABs 90, 91, 92, 93, and 94) produced specifically to this allergen and another MAB 12 that was found to cross-react with *Lol p IV*, two distinct epitopes, A and B, have been identified

on *Lol p IV* (Fig. 3). However, the possibility that the fine specificities of MAbs 90 to 94 differed from one another cannot be completely ruled out. For example, it has been reported that protein antigens, such as the influenza virus hemagglutinins, have numerous epitopes, which are clustered at major antigenic sites. In these situations, it may be difficult, if it is not impossible, to resolve differences at such sites. It was through the use of closely related, cross-reacting species of influenza virus that the differences in specificity of the MAbs and, consequently, differences in epitopes were revealed.^{14, 15} A similar approach with cross-reacting group IV allergens isolated from other related grass pollens may help to reveal any differences in the specificity of the epitopes recognized by the MAbs 90 to 94.

Sites A and B were also considered to constitute at least two of the allergenic sites of *Lol p IV* because all the MAbs could compete with polyclonal human IgE antibodies and inhibited the binding of ¹²⁵I-labeled *Lol p IV* to the human IgE antibodies (Table I). It was clear that these two allergenic site(s) recognized by human IgE antibodies were either identical or at least very close to the antigenic sites A and B recognized, respectively, by MAbs 90 to 94 and 12 used in this study. The low degree of inhibition observed with MAb 94 was consistent with the suggestion that this MAb has a low binding affinity to *Lol p IV* (Fig. 2).

Major differences in the repertoire of allergenic specificities recognized by allergic patients was demonstrated by the finding that the serum of patient A. E. lacked IgE antibodies to site A. Other investigators have also observed differences in the degree to which human IgE antibodies and MAbs compete for binding to an allergen. For example, Chapman et al.¹⁶ found that the binding of MAbs directed to two distinct epitopes on allergen *Der p I* of *Dermatophagoides pteronyssinus* could not be inhibited by human IgE antibodies, whereas Mazur et al.¹⁷ found that the binding of human IgE antibodies to an insect allergen was inhibited to a maximum of 15% by MAbs. In the present study, the degree to which the MAbs inhibited the binding between *Lol p IV* and IgE was of a similar order to that observed between human IgE antibodies and *Lol p I*¹³ or the group I allergens of other grasses.¹⁸ Olson and Klapper¹⁹ observed that MAbs directed to two different sites on *Amb a I* of ragweed pollen could inhibit the binding of human IgE antibodies to *Amb a I* up to 50%.

Allergen-specific human serum IgG antibodies have been reported to be directed to the antigenic/or allergenic site(s) recognized also by MAbs.^{13, 16} In the present investigation, all sera from atopic subjects

were found to possess IgG antibodies to *Lol p IV* (Fig. 4) in addition to anti-*Lol p IV* IgE antibodies. Two sera from nonatopic subjects (K. J. and T. C.), devoid of IgE antibodies to *Lol p IV*, were nevertheless found to possess IgG antibodies to this allergen. Their specificity was determined indirectly in terms of the degree to which the human sera could compete with and inhibit the binding of site A- and site B-specific ¹²⁵I-labeled MAbs to *Lol p IV*. Clearly, regardless of their isotype, any antibodies directed to *Lol p IV* could participate in inhibiting the binding of the MAb to *Lol p IV*. Thus, all the sera (except from T. C.) had antibodies to site A. Similarly, it was found that all sera (except from D. B. and J. Y.) had antibodies to site B (Table II). These observations revealed that the human sera contained, in addition to IgE antibodies to sites A and B, also antibodies of other isotypes that recognized the same or similar epitopes on *Lol p IV*.

Despite the fact that the allergic human sera from D. B. and J. Y. possessed IgE antibodies, some with specificities to *Lol p IV* recognized by MAb 12 (Table I), it was surprising to find that sera from D. B. and J. Y. were unable to compete with a radiolabeled preparation of MAb 12 for binding to *Lol p IV* (Table II). The reason for this apparent discrepancy is not known at this time.

In our continuing attempts to identify the repertoire of epitopes on *Lol p IV*, nine additional MAbs recently produced against *Lol p IV* were all found to be directed to site A. It is suggested therefore that site A is recognized by BALB/c mice as the immunodominant determinant of *Lol p IV*. Observations of a similar nature have been reported for *D. pteronyssinus*¹⁶ and *Amb a I*¹⁹ in which most (60% to 70%) of the corresponding MAbs were respectively directed to a single epitope.

Two-site radiometric assays with the same MAbs for preparation of (1) the solid phase capable of binding the allergen and (2) the radiolabeled indicator antibody have demonstrated the presence of nonrepeating epitopes on *Amb a I*¹⁹ and *Der p I*.²⁰ However, in the present study, none of the MAbs (with the exception of MAb 12) could be successfully coated onto microtiter plates to prepare the solid phase. A radiolabeled preparation of MAb 12 exhibited an unacceptably high level of nonspecific binding to the solid phase. Thus, it was not possible to perform the two-site assay with our reagents. Similar difficulties in preparing solid phase with MAbs have been noted by other investigators.^{21, 22}

The identification and characterization of the different epitopes on *Lol p IV* will help in understanding the overall structure of this allergen. It has been dem-

onstrated that MAbs also offer a useful tool in the isolation of purified allergens from crude allergen extracts by a single adsorption on MAb-coupled affinity chromatography columns.^{12, 23} We have earlier reported that *Lol p* IV can be purified to apparent homogeneity by this method from crude ryegrass extract with MAb 12-coupled Sepharose affinity chromatography.¹⁰ The low affinity of MAbs 12 and 94 demonstrated in the present study makes them an ideal ligand for this type of purification procedure.

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