

STUDY OF THE EPITOPE STRUCTURE OF PURIFIED DAC G I AND LOL P I, THE MAJOR ALLERGENS OF DACTYLIS GLOMERATA AND LOLIUM PERENNE POLLENS, USING MONOCLONAL ANTIBODIES

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The use of mAb allowed us to further analyze the cross-reactivity between purified Dac g I and Lol p I, the major allergens of *Dactylis glomerata* (cocksfoot) and *Lolium perenne* (Rye grass), respectively. It was first shown, using IEF, followed by immunoprinting, that serum IgE antibodies from most grass-sensitive patients recognize both Dac g I and Lol p I. Second, three different anti-Lol p I mAb, 290A-167, 348A-6, and 539A-6, and one anti-Dac g I mAb, P3B2 were all shown to react with Dac g I and Lol p I, indicating that the two molecules share common epitopes. Epitope specificity of the mAb was determined by competitive binding inhibition of a given labeled mAb to solid phase fixed Dac g I or Lol p I by the mAb. The results indicated that the four mAb are directed against four different and non-overlapping epitopes present on both allergens. Using double-binding RIA, our data strongly suggest that the common epitopes are not repetitive on both molecules. In addition to their similar physico-chemical characteristics, such as isoelectric points and m.w., Dac g I and Lol p I share four identical epitopes. Binding inhibition of human IgE to Lol p I and Dac g I by the mAb was also assessed. The results indicated that each mAb was able to inhibit such reactions to variable degree but no additive inhibition was observed when two mAb of different specificities were used in combination, suggesting that the human IgE binding site is partially shared by each epitope recognized by the four mAb.

The identification of grass pollen allergens, especially the major allergens, is of prime importance in the diagnosis, treatment, and study of allergic diseases. Serum IgE antibodies (Ab)² from grass-sensitive patients can be used to detect cross-reactivity between allergens but cross-sensitization by other allergens cannot be ruled out. mAb (1) against well defined epitope are probably

best to assess the presence of specific and common antigenic determinants borne by different molecules. Several mAb against grass pollen allergens have been produced to perform standardization studies (2), allergen purification (3-5), and to investigate the cross-reactivity between grass pollen allergens (4-6). Recently, it has been reported that murine mAb could inhibit the binding of human IgE Ab to specific allergens and this observation leads to the conclusion that both Ab (human IgE and mAb) can be directed against identical or closely related allergenic epitopes (7-10).

Inasmuch as Dac g I and Lol p I, the major allergens of *Dactylis glomerata* (11) and of *Lolium perenne* pollens respectively (12, 13) and their respective mAb are available and well defined, we further studied the characterization of epitopes common to Dac g I and Lol p I. We also aimed to define the specificity of those murine mAb as compared to human IgE Ab from grass-sensitive patients toward Dac g I and Lol p I.

MATERIALS AND METHODS

Allergen extracts and purified allergens. *Dactylis glomerata* (Cocksfoot) pollen was obtained from the Pasteur Institute (Paris, France) and SE was prepared as described by Mecheri et al. (14) The major allergen (Dac g I) of this SE was purified as already described (11). Soluble extracts of *Lolium perenne* were obtained from Omega Laboratories (Montreal, Canada) and Lol p I was purified on a reverse immunosorbent column (5).

Patient sera. Patients with a history of classical allergic rhinitis to grass pollen who were followed at the Pasteur Institute Hospital and who had positive skin tests (prick technique) to *Dactylis glomerata* and *Lolium perenne* pollens were enrolled in this study. Control subjects with no history of allergic rhinitis and negative skin tests to all grass pollens were also included.

Detection of specific IgE for the SE constituents in sera of allergic patients. The detection of specific IgE Ab to the SE constituents of the two grass pollens was performed as described by Peltre et al. (15). Briefly, the pollen constituent of *Dactylis glomerata* and *Lolium perenne* separated by IEF from two granulated agarose gels were transferred to separate nitrocellulose sheets. Then strips of these prints were incubated overnight with 200 μ l of sera from different *Dactylis glomerata* and *Lolium perenne* grass-sensitive patients diluted with 400 μ l PBS containing 0.1% Tween 20. After extensive washes with saline, each strip was incubated for 2 h with ¹²⁵I-labeled anti-human IgE (RAST-kit; Pharmacia, France). After B015 D' Arc4 washes autoradiography was carried out using Kodak-X-Omat film at -70°C with a Cronex (Dupont de Nemours Montreal, Canada) Intensifier Screen.

Cell fusion, cloning, and production of mAb against Dac g I and Lol p I allergens. Spleen cells from BALB/c mice immunized either with purified Dac g I or Lol p I were fused separately with non-secreting SP2-0 myeloma cells in the presence of polyethylene glycol according to Kohler and Milstein (1). One mAb (P3B2) against Dac g I allergen (4) and three others (290A-167, 348A-6, and 539A-6) against Lol p I were obtained (5). The purification of mAb P3B2 was carried out by precipitation of the ascitic fluid with a Na₂SO₄ solution

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² Abbreviations used in this paper: Ab, antibody; Lol p I, the new nomenclature of rye group I; pl, isoelectric point; SE, soluble extracts; Dac g I, the new nomenclature of *Dactylis glomerata* group I; cpm, counts/min.

(18%). The supernatant was discarded and the pellet was dissolved in saline and dialyzed against large volumes of phosphate buffer (pH 6.2, 0.0175 M). The purification of IgG antibodies was carried out by ion exchange liquid chromatography (16). The proteins were loaded onto a DEAE-cellulose column (DEAE-52, Whatman Inc., Clifton, NJ) previously equilibrated with PBS (0.0175 M, pH 6.3). The IgG fraction containing mAb P3B2 was obtained at the isocratic elution step.

Anti-Lol p I mAb were purified by passing the corresponding ascitic fluids through a protein A-Sepharose 4B column. Antibodies were then eluted, dialyzed against PBS, and stored at -80°C until use.

ELISA. To determine if Dac g I and Lol p I share common epitopes, ELISA experiments were performed in 96-well microtiter plates using the technique of Voller et al. (17). Briefly, 0.1 ml of either Dac g I or Lol p I allergens at 1 $\mu\text{g}/\text{ml}$ in sodium carbonate buffer (pH 9.5, 0.05 M) were incubated for 2 h at 37°C . Unbound material was removed by extensive washes with PBS-Tween-20 0.1%. The plates were then saturated with 3% BSA for 1 h at 37°C . Various concentrations (20 to 0.009 $\mu\text{g}/\text{ml}$) of the different mAbs (100 $\mu\text{l}/\text{well}$) were added to Dac g I or Lol p I coated wells for 2 h at 37°C . After three washes, 100 $\mu\text{l}/\text{well}$ of horseradish peroxidase-conjugated sheep anti-mouse IgG Ab (1/1000, N. L. Cappel Laboratories, Cochranville, PA) were added and incubated for 2 h at 37°C . After extensive washes, 100 μl of o-phenylenediamine (0.4 mg/ml) in citrate buffer (pH 4.5, 0.05 M) containing 0.006% H_2O_2 were added. The reaction was stopped after 5 min by the addition of 50 μl of 4 N H_2SO_4 and the OD read at 492 nm with an automatic spectrophotometer.

Radiolabeling of purified mAb. A total of 20 μg of purified anti-Lol p I mAb (290A-167, 348A-6, and 539A-6) and anti-Dac g I mAb (P3B2) and mAb anti-human IgE (7H8) were iodinated with 200 μCi of ^{125}I (Amersham Corp., Arlington Heights, IL) by using Iodo-Gen (Pierce Chemical Co., Rockford, IL). Free iodine was separated from radiolabeled proteins by passage through a Sephadex G-50 column. Specific activities were estimated following the instructions of the company, and were in the range of 5 to 12×10^6 cpm/ μg .

Epitope specificity of anti Lol p I and anti Dac g I mAb. The epitope specificity of mAb was analyzed by competitive binding to polystyrene bound Dac g I and Lol p I of radiolabeled anti-Dac g I or anti-Lol p I mAb and either homologous unlabeled mAb or unrelated mAb. Polystyrene removable strips with flat-bottom wells were coated with 100 μl containing 20 ng of Dac g I or Lol p I diluted in sodium carbonate buffer (0.05 M, pH 9.6). After a 2 h incubation at 37°C , free protein-binding sites were saturated with 200 μl of 3% BSA per well. After washing, each ^{125}I -labeled mAb (10^5 cpm/well) was added alone (100% binding) or in the presence of homologous or unrelated unlabeled mAb used as inhibitors, to a final volume of 100 μl . Inhibitors were used at concentrations ranging from 2 to 0.15 $\mu\text{g}/\text{ml}$. After a 2 h incubation at 37°C , wells were washed and radioactivity was counted in an LKB gamma counter and the results were expressed as a percentage of inhibition calculated as follows:

$$\left(1 - \frac{\text{cpm bound in presence of inhibitor}}{\text{cpm bound in absence of inhibitor}}\right) \times 100$$

Double-binding RIA. These experiments were designed to determine if the epitopes recognized by mAb P3B2 and anti-Lol p I mAb are present only once or several times on Dac g I and Lol p I allergens. Polystyrene removable strips (Dynatech Laboratories Inc., Alexandria, VA) were first coated with 100 μl of 1 $\mu\text{g}/\text{ml}$ of mAb P3B2 or anti-Lol p I mAb diluted in sodium carbonate buffer (pH 9.5, 0.05 M) followed by 200 μl of 3% BSA/well to saturate additional protein-binding sites. Wells were then washed with PBS-Tween 20 (0.1%) and 100 μl (1 $\mu\text{g}/\text{ml}$) of Dac g I or Lol p I were added. These Ag were diluted in the washing buffer containing 10 mM of EDTA to prevent any possible aggregation of the Ag. After overnight incubation at room temperature, unfixed allergens were removed by extensive washes and 100 μl of ^{125}I -labeled mAb (10^5 cpm/well) were added and incubated for 3 h at 37°C . Then, after washes, radioactivity was counted in a LKB gamma counter.

Specificity of human IgE Ab. The capacity of mAb to inhibit the binding of human IgE Ab to the allergens was assessed. Polystyrene removable strips were coated with either Lol p I or Dac g I (200 ng/well) and saturated with 3% BSA. The plates were washed with PBS and incubated overnight with human serum (pool from grass-sensitive patients; final dilution 1/4) alone or in the presence of one or two of the mAb (290A-167, 348A-6, 539A-6, or P3B2) at a final concentration of 5 $\mu\text{g}/\text{ml}$ each. A mAb (13A-12) directed against an unrelated Ag was used as negative control. After washing, labeled anti-human IgE mAb (7H8) was added (10^5 cpm/well/100 μl) and incubated for 18 h. Wells were then extensively washed, the radioactivity counted, and the percentage of inhibition calculated.

RESULTS

Heterogeneity of allergens recognized by serum IgE antibodies from patients sensitive to grass pollen. Sera from 17 grass-sensitive patients were investigated for their ability to recognize allergenic components of the pollen extracts by the immunoprint technique. A pool of normal human sera was included as a control. The different constituents of the two pollen SE were separated by IEF in an agarose gel and, as shown in Figure 1, at least 50 bands with pI ranging from 3.5 to 10.5 were visualized by India ink staining.

The print of each pollen SE was made on a nitrocellulose sheet that was cut into 18 strips and each strip was incubated with a serum from a single patient. Serum IgE antibodies bound to the different constituents of the pollens were identified by exposure to ^{125}I anti-human IgE followed by autoradiography. Comparison with the complete pattern of protein components shown on the right of the upper part of Figure 1 makes it clear that constituents are not all allergenic and that a marked difference was observed from one patient to another. The majority of the patients' sera (90%) recognized the major allergen (Dac g I) with a pI of 5.9 (upper panel, Fig. 1).

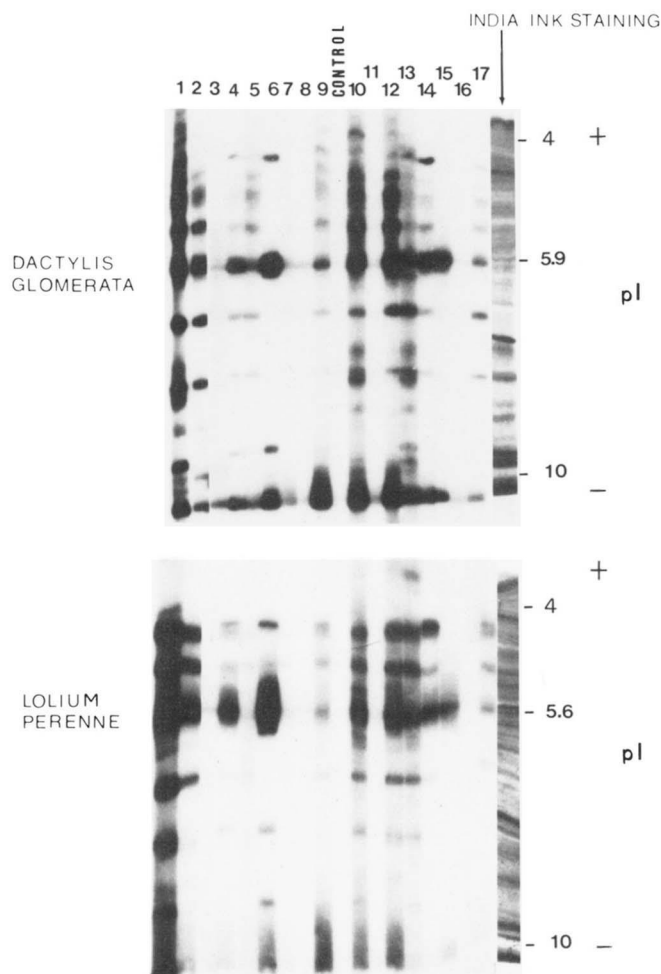


Figure 1. Heterogeneity of the *Dactylis glomerata* and *Lolium perenne* pollen allergens as shown in immunoblot with IgE antibodies from 17 grass-pollen-sensitive patients. A pool of normal patient sera was also used as a negative control. The bands on the right of the upper and the lower panels show the heterogeneity of *Dactylis glomerata* and *Lolium perenne* grass pollens, respectively, stained by India ink with pI ranging from 3.8 to 10.5.

Another allergen with a pI around 10 was recognized by the patients sera with the same frequency as Dac g I. The immunoblot profile obtained with *Lolium perenne* pollen extracts was comparable to that of *Dactylis glomerata* using the same sera (lower panel, Fig. 1). Again, one band is recognized by the serum IgE from most patients (80%). This allergenic component with a pI of 5.3 to 5.6 seems to be Lol p I, the major allergen of this pollen. The profiles of reactivity in immunoblot with human sera is comparable for the two pollens studied, and it became tempting to study any possible cross-reactivity between the two pollen extracts at the molecular level using the purified major allergens: Dac g I and Lol p I.

Cross-reactivity between Dac g I and Lol p I. To investigate if Dac g I and Lol p I share common epitopes, the anti Lol p I mAb, 290A-167, 348A-6, and 539A-6 were tested for their capacity to interact either with Dac g I or with Lol p I. Conversely, the anti-Dac g I mAb, P3B2, was tested for its binding capacity to Dac g I and to Lol p I. The binding assay was carried out by ELISA using a solid phase-bound allergen. As shown in Figure 2, both allergens were recognized by anti-Lol p I and anti-Dac g I mAb. The mAb bound to immobilized allergens in a dose-dependent fashion. It is striking to observe that each mAb reacted with Dac g I and Lol p I to the same extent. This observation suggests that the two molecules could display similar epitopes. However, the binding patterns of the three anti-Lol p I mAb were comparable (Fig. 2B, C, and D) as compared to anti-Dac g I mAb P3B2 (Fig. 2A). These results may be explained by the fact that the

mAb were purified from ascitic fluids either by DEAE chromatography or by using protein A affinity column; therefore, each preparation contains both mAb and normal murine IgG and the amount of mAb in the purified samples could differ. This cross-reactivity is restricted to grass pollens because no binding of any of the mAb to AgE, the major determinant of ragweed, was seen (not shown).

Specificity of different mAb. As previously reported, using competitive binding to polystyrene-bound Ag, the three anti-Lol p I mAb recognize distinct epitopes of the Lol p I molecule (18). Our experiments were designed to investigate the relationship between the epitope recognized by mAb P3B2 and those interacting with anti-Lol p I mAb. An RIA was performed involving a binding inhibition of radiolabeled anti-Lol p I mAb to Lol p I either by homologous unlabeled mAb or by unlabeled mAb P3B2. As shown in Figure 3, a specific inhibition was observed, whereas no inhibition was obtained with mAb P3B2, which suggests that the three epitopes on Lol p I that are recognized by the three anti-Lol p I mAb are distinct from the one recognized by P3B2.

In another set of experiments, binding specificity of the four mAbs to their specific epitopes on Dac g I was also demonstrated by binding inhibition. Figure 4 displays a specific binding of the four mAb inasmuch as only homologous mAb was capable of inhibiting binding to Dac g I in a dose-dependent manner. These data suggest that the Dac g I epitopes recognized by the four mAb are structurally independent.

Representativity of common epitope of Dac g I and

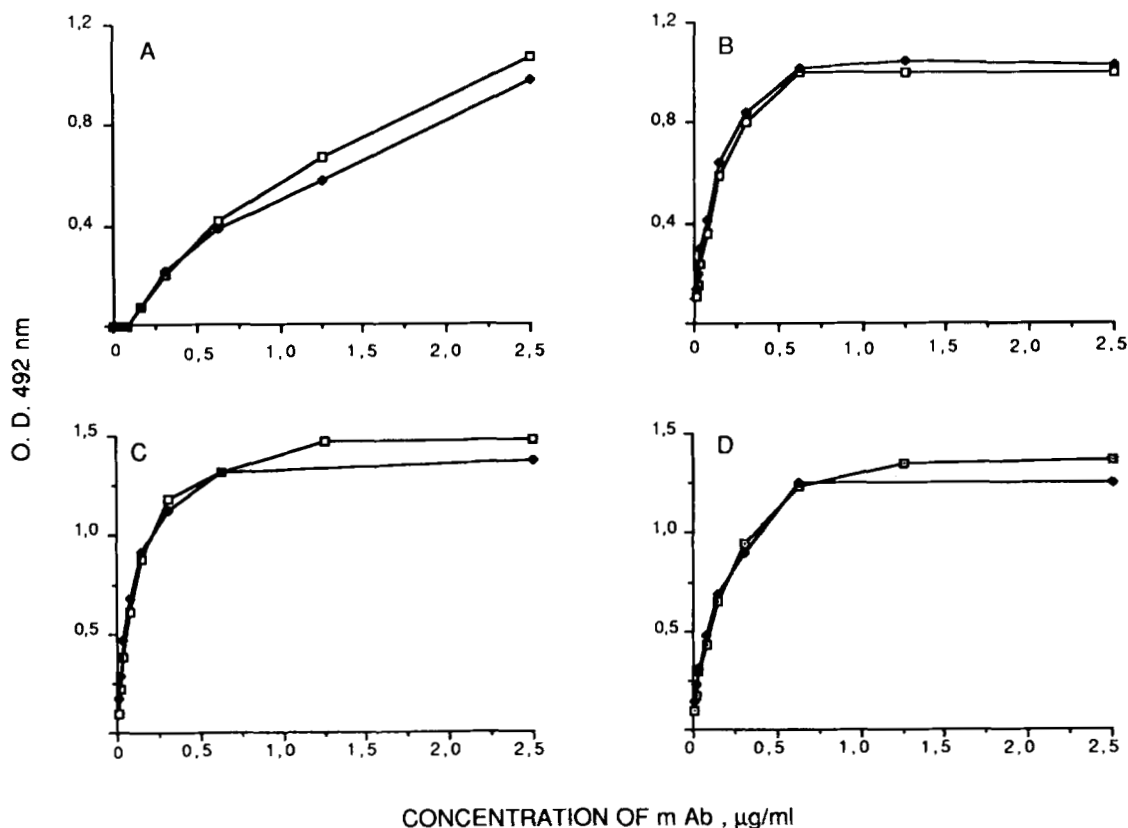


Figure 2. Binding curves of the reaction between immobilized Dac g I (□) or Lol p I (●) and P3B2 (A), 290A-167 (B), 348A-6 (C), and 539A-6 (D) mAb. Different concentrations of mAb were added in triplicate to allergen-coated wells (1 µg/ml). The mAb binding was revealed by adding horseradish peroxidase-conjugated sheep anti-mouse IgG antibodies.

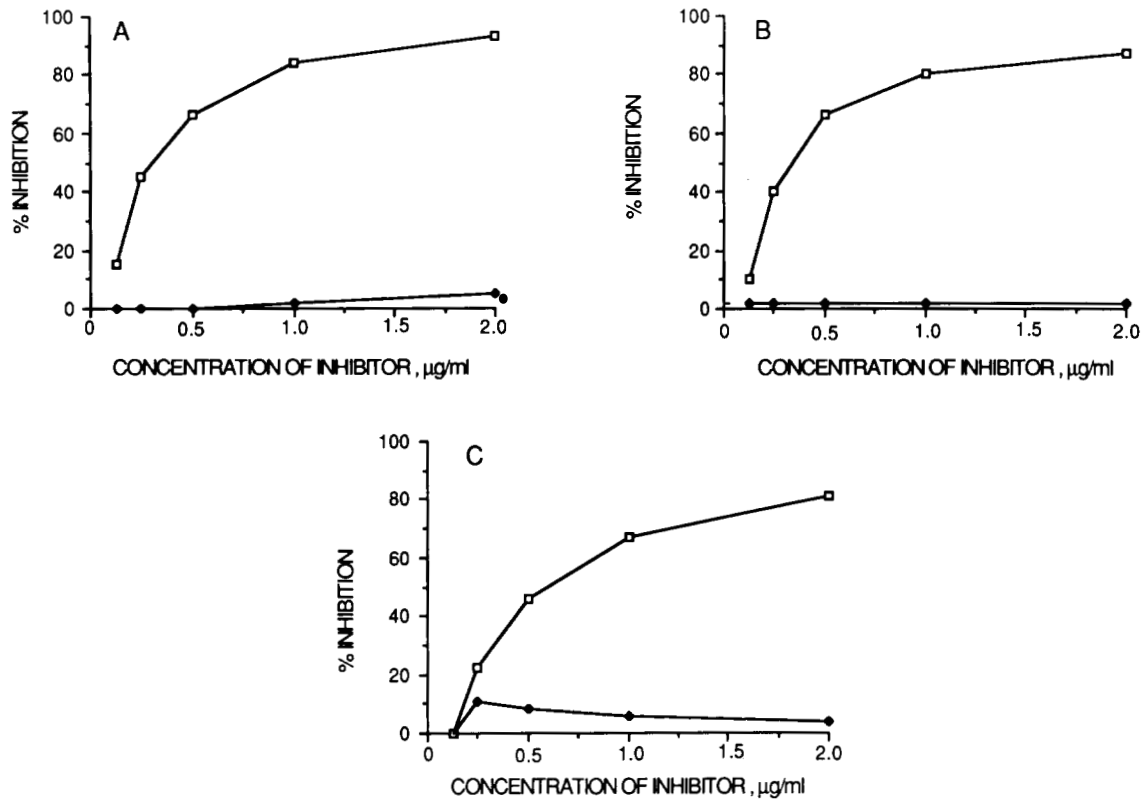


Figure 3. Binding inhibition of ^{125}I -labeled anti-Lol p I mAb (290A-167, A; 348A-6, B; and 539A-6, C) to solid phase Lol p I used alone (□) or in combination with P3B2 anti-Dac g I (◆) at different concentrations. Each point represents the mean value of three experimental determinations.

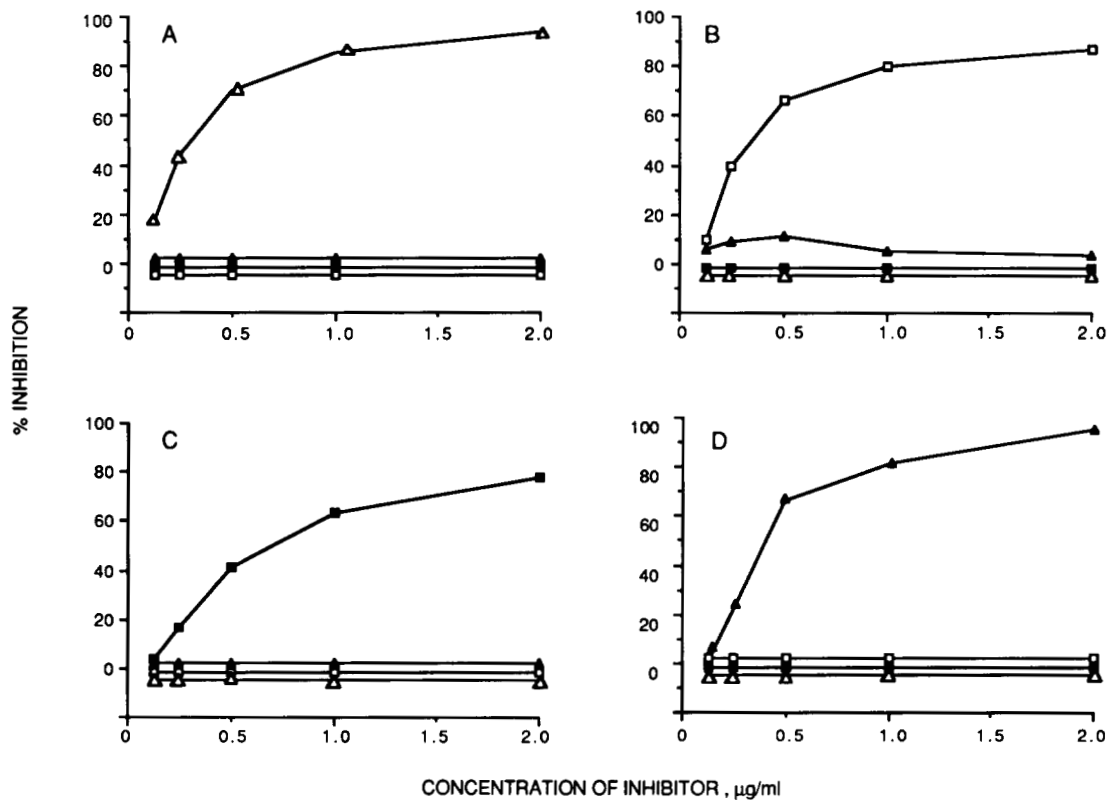


Figure 4. Binding inhibition of ^{125}I -labeled mAb (290A-167, A; 348A-6, B; 539A-6, C; and P3B2, D) to solid phase Dac g I by unlabeled mAb (290A-167, △; 348A-6, □; 539A-6, ■; and P3B2, ▲). Each point represents the mean value of three experimental determinations.

Lol p I on the two molecules. To determine if the four different common epitopes are equally represented on the two allergens, a double-antibody RIA was done. Dac

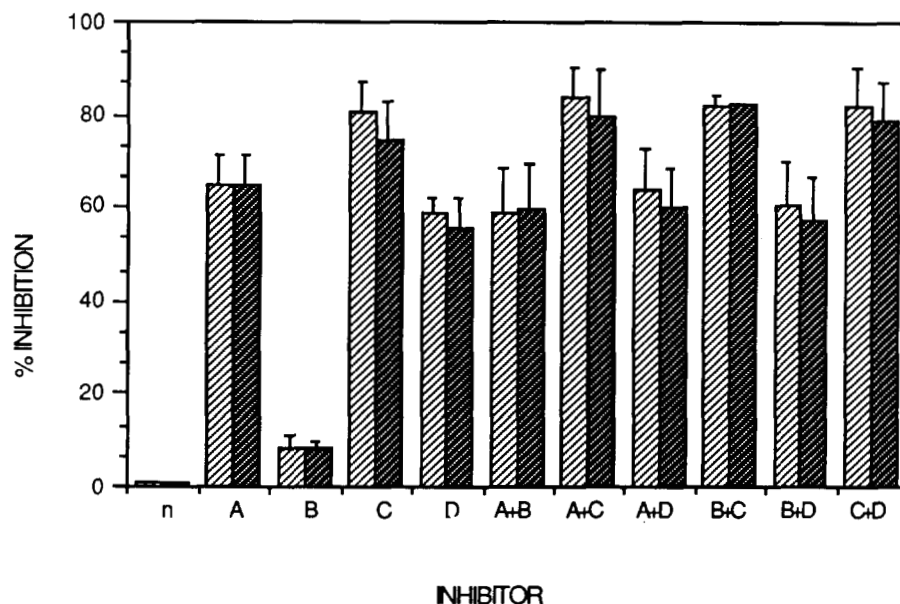
g I and Lol p I allergens were allowed to react with polystyrene-bound mAb and either with related or unrelated radiolabeled mAb. The results of this assay are

TABLE I
Binding of labeled mAb to Dac g I and Lol p I presented by unlabeled mAb^a

Ag	Labeled mAb							
	P3B2		290A-167		348A-6		539A-6	
	Dac g I	Lol p I	Dac g I	Lol p I	Dac g I	Lol p I	Dac g I	Lol p I
Solid phase fixed mab	(cpm)							
P3B2	860	218	14825	15534	36582	5060	61421	20630
290A-167	19827	4478	2680	1566	29625	71186	54622	73901
348A-6	21721	10732	15618	69931	786	800	59623	70809
539A-6	26314	34894	12617	84812	32723	64332	3826	2698

^a The values shown are the mean of three experimental determinations.

Figure 5. Binding inhibition of human IgE mAb to Lol p I (light bars) and Dac g I (dark bars) by mAb (290A-167, A; 348A-6, B; 539A-6, C; and P3B2, D) used alone or in combination. The final concentration of mAb used in this assay was 5 μ g/ml.



shown in Table I. The binding of the labeled mAb to Lol p I or Dac g I molecules bound to homologous immobilized mAb was not significant. This observation could suggest that specific epitopes on both molecules, when bound to fixed mAb, are not available for further binding with the free labeled mAb. Comparable results were obtained even if the immobilized mAb was diluted 10 fold (not shown). In contrast, when the labeled mAb differs from the immobilized one, the labeled mAb could bind to the molecule without interference, suggesting that their specific epitopes seem to be free and not modified by binding of the molecule to a given mAb. This suggests that all epitopes are not repeated and are present only once on each molecule of Lol p I and Dac g I.

Binding inhibition of human IgE Abs to Dac g I and Lol p I by mAb. The capacity of mAb to inhibit the reaction between human IgE Ab and the allergens was assessed. The binding of IgE Ab to both allergens could be significantly inhibited by mAb 539A-6, 290A-167, and P3B2 and to a lesser degree by mAb 348A-6 (Fig. 5). These results were obtained at concentration of 5 μ g/ml for each mAb. This concentration was, in preliminary experiments, determined as optimal to achieve maximal inhibition of the same Ab without affecting the binding of different mAb. Combination of two inhibitors did not yield additive inhibition. Also, inhibition was studied using lower concentration of mAb in order to decrease the possibility of steric hindering and no additive inhibition was observed (not shown). This suggests that the human IgE binding site on both molecules is partially shared by each epitope recognized by the four mAb.

DISCUSSION

In this work, we took advantage of the availability of mAbs to study the cross-reactivity between the two major allergens (Dac g I and Lol p I) of *Dactylis glomerata* and *Lolium perenne*. We first showed that serum IgE Ab from individuals sensitive to grass pollen react with different components of the SE from the two pollens, among which the major allergens, as based on physical properties previously described (4, 5, 19, 20). These were recognized with high frequency (80 to 90%), suggesting that they are indeed the major allergens according to the definition of Marsh (13). In addition to Dac g I and Lol p I, it is striking to note that allergenic determinants recognized by a given patient's serum are comparable in both *Dactylis glomerata* and *Lolium perenne* pollen extracts. It is tempting to believe that cross-reactivity between the two pollen extracts is not restricted to their major allergens, but that many other allergens are also involved.

The difficulties encountered with serum polyclonal antibodies in studying cross-reactivity between allergens are greatly alleviated with the use of mAb. The fact that the major allergens, Dac g I and Lol p I, are both recognized by mAbs directed against Dac g I provides further evidence that these allergens have common antigenic determinants (4). We have extended these findings showing that antigenic epitopes common to Dac g I and Lol p I are recognized by anti-Lol p I (290A-167, 348A-6, and 539A-6) mAb as shown by solid-phase ELISA where immobilized allergens were exposed to different concentrations of mAbs (Fig. 2). Binding curves obtained with each

mAb displayed similar profiles for Dac g I and Lol p I.

The epitope specificity of the three anti-Lol p I mAb (290A-167, 348A-6, and 539A-6) on the Lol p I has been previously characterized (18) and in this work we compared their specificity to those of mAb P3B2 on the Lol p I and Dac g I molecules. Our data showed that the Lol p I epitope recognized by P3B2 mAb is distinct from those recognized by the three anti-Lol p I mAb. Furthermore, all four mAb are directed against four independent and non-overlapping Dac g I epitopes. Taken together, these results underline the high degree of homogeneity between the two molecules, but do not provide information on the quantitative representation of each epitope. However, the results of the double binding RIA suggest that the four epitopes are present only once on each molecule of Lol p I or Dac g I.

Binding inhibition of human IgE to the allergens is a classical assay used to compare the specificity of mAb and human IgE Ab. Previously, other investigators using such an assay to study different Ag (8–10) have reported that indeed mAb and human IgE Ab could recognize the same epitope. Our data showed that mAb strongly inhibit the binding of human IgE to Lol p I and Dac g I. However, no additive inhibition of human IgE binding was observed when a combination of two murine mAb of different specificities was used. Additive inhibition is a necessary, but not always sufficient condition to conclude that human IgE Ab and murine mAb are directed against the same epitope. Indeed, an additive inhibition could be observed if two distinct IgE binding sites of the allergens are, each of them, partially recognized by one mAb. Therefore, determination of the amino acid sequences is absolutely necessary to draw such a conclusion. In our hands, the epitopes recognized by the four mAb are non-overlapping and the combination of two mAb did not yield to any additive inhibition of human IgE binding. These observations suggest that the site where human IgE binds to the allergen is partially shared by the epitopes recognized by the four mAb. The high level of inhibition (80.2% obtained by 539A-6 mAb) does not necessarily mean that 80.2% of the IgE are directed against one epitope partially shared by mAb and human IgE. It is possible that other sites reacting with human IgE Ab are not well exposed to their specific IgE Ab because of their attachment to solid matrix (10).

Results herein provide evidence that Dac g I and Lol p I share four common and independent epitopes and these observations do not rule out the possibility of other specific or cross-reacting epitopes on Lol p I and Dac g I. Human IgE Ab could be directed against an epitope which is partially shared by different mAb and it would be of great interest to test mAb produced in other laboratories to gather more information on the allergenic structure of Dac g I and Lol p I. Further studies are needed to better

characterize the structural relationship between Dac g I and Lol p I and to compare the specificity of mAb and human IgE Ab.

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