

IMMUNOCHEMICAL STUDIES OF *Lolium perenne* (RYE GRASS) POLLEN ALLERGENS, *Lol p* I, II, AND III^{1,2}

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It was reported earlier that human immune responses to three perennial rye grass (*Lolium perenne*) pollen allergens, *Lol p* I, II, and III, are associated with histocompatibility leukocyte antigen (HLA)-DR3. Rye-allergic people are often concordantly sensitive to all three of these allergens. Since earlier studies suggested that these antigens are non-cross-reactive, their immunologic relatedness by double antibody radioimmunoassay (DARIA) was studied in order to understand further the immunochemical basis for the concordant recognition of the three allergens. Direct binding DARIA studies were performed with human sera from 189 allergic subjects. Inhibition DARIA studies were carried out with 17 human sera from grass-allergic patients who were on grass immunotherapy, one goat antiserum, and six rabbit antisera. None of the sera detected any significant degree of two-way cross-reactivity between *Lol p* I and II, or between *Lol p* I and III. However, the degree of two-way cross-reactivity between *Lol p* II and III exhibited by individual human and animal antisera varied between undetectable and 100%. In general, the degree of cross-reactivity between *Lol p* II and III was higher among human sera than among animal sera. Taken together with earlier findings that antibody responses to *Lol p* I, II and III are associated with HLA-DR3, and that most *Lol p* II and III responders are also *Lol p* I responders, but not vice versa, our present results suggest the following: the HLA-DR3-encoded Ia molecule recognizes a similar immunodominant Ia recognition site (agretope) shared between *Lol p* I and *Lol p* II and/or III; in addition, *Lol p* I appears to contain unique Ia recognition site(s) not present in *Lol p* II and III. However, further epitope analyses are required to investigate these possibilities.

Since the early studies of Wyman (2), it has been frequently noted that allergic subjects often exhibit some degree of specificity in their clinical symptomatology (e.g., grass vs ragweed pollen sensitivity). During the course of

our previous studies of specific immune responses to individual allergens isolated from grass and ragweed pollens (3-9), we have noted that many subjects showing selective clinical symptoms to a particular pollen species have IgE and IgG serum antibodies (Ab)⁴ to several immunochemically distinct allergens from the same species, but not to other allergens of taxonomically unrelated species. This finding suggests some unsuspected chemical relatedness between certain allergens from the same species which might account for this concordant responsiveness.

A particular example of this type of concordant responsiveness is to *Lol p* I, II, and III (Rye I, II, and III) allergens of *Lolium perenne* (perennial rye) grass pollen (8, 9). These proteins were previously thought to be non-cross-reactive antigenically and allergenically (10-15). However, it has been noted that subjects allergic to *Lol p* II (m.w. 11,000) and *Lol p* III (m.w. 11,000) are usually also allergic to *Lol p* I (m.w. 27,000), and that log [IgE Ab] or log [IgG Ab] levels to *Lol p* I and *Lol p* II are significantly correlated (8). We also found that immune responsiveness to all the three allergens is significantly associated with histocompatibility leukocyte antigen (HLA)-DR3 (9).

These findings suggest that the primary major histocompatibility complex-restricted immune recognition of the three *Lol p* allergens may involve the interaction of the same, or a related, Ia antigenic site (agretope) of the three antigens with a common Ia molecule. However, another explanation is that concordant responsiveness to all three allergens results from an unsuspected antigenic cross-reactivity between them. In order to investigate this matter further, we have studied cross-reactivities between the three allergens.

MATERIALS AND METHODS

Study groups. Sera from two groups of Caucasoid human subjects were used. Group A consisted of 189 employees of the Westinghouse Electric Corporation, Hunt Valley, Baltimore County, MD (3-5). The group included 122 subjects who were skin test positive (ST⁺) to *L. perenne* extract (LPE); and 67 subjects who were ST⁻ to LPE, but ST⁺ to one or more of certain other common inhalant allergens (extracts of short ragweed and timothy grass pollen, cat dander, house dust, and *Alternaria* spp.). Group B was a selection of 17 individuals from our clinic subjects (6). A total of 19 serum samples from this group were used (one bleed each from 16 of the subjects, and three bleeds from the 17th subject). These selected individuals had all received grass immunotherapy. Thirteen of the sera had high levels of IgG Ab to all the three allergens; three sera had IgG Ab to *Lol p* I and III, but little or no detectable Ab to *Lol p* II; and three sera had IgG Ab only to *Lol p* I with no detectable Ab to *Lol p* II or III.

Animal antisera. Goat anti-LPE antiserum G797-9 (NIH Research Resources Reagent No. A-617-601-557) was a pool of high-titer sera from three goats that were each hyperimmunized with repeated,

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² *Lol p* I is the designation given under the new IUIS Allergen Nomenclature system (1).

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⁴ Abbreviations used in this paper: Ab, antibody; DARIA, double-antibody radioimmunoassay; LPE, *Lolium perenne* extract.

multiple-site intramuscular injections of 2 to 10 mg dialyzed LPE emulsified with Freund's complete adjuvant containing 10 mg/ml *Mycobacterium butyricum*. Rabbit serum pool R201-3 was produced in a similar fashion by hyperimmunizing three rabbits with dialyzed LPE. Rabbit sera R69 and R100 were prepared by immunization of individual rabbits every other week, in multiple sites, with partially purified *Lol p I* (1 to 2 mg) emulsified in Freund's complete adjuvant; rabbit serum R67 was prepared by using a similar immunization schedule with partially purified *Lol p II*. Sera R107 and R138 were prepared by immunizing rabbits with partially purified *Lol p I* (2 mg) adsorbed on alumina gel adjuvant.

Rye allergens. *Lol p I* and *II* were purified by using a modification of the procedure described earlier (8). Briefly, *L. perenne* pollen (Greer Labs., Lenoir, NC) was defatted, extracted (16), and successively chromatographed on DEAE-cellulose and DEAE-Fractogel 650 (S) columns. The fraction containing *Lol p I* and *II* was passed through a Sephadex G-75 (superfine) column to separate the two major components. The *Lol p II* fraction was further purified on DEAE-Sephadex A-25 column using a 0.08 M to 0.2 M gradient of ammonium bicarbonate-ammonium hydroxide (pH 8.4). The *Lol p I* fraction obtained from the Sephadex G-75 run was also run through a DEAE-Sephadex A-25 column by using a 0.08 M to 0.2 M gradient of ammonium bicarbonate-ammonium hydroxide (pH 8.4). Further purification of *Lol p I* was achieved by high performance liquid chromatography on a Waters' DEAE 5PW, 7.5 mm $\times$ 7.5 cm column.

Lol p III was purified as follows: high m.w. proteins were removed from LPE by passing it through an Amicon hollow fiber cartridge H1P5 with a nominal cutoff of 5,000; we have observed that this cartridge allows the passage of cytochrome *c* (17) and *Lol p III*. The ultrafiltrate containing *Lol p III* was concentrated with the use of an Amicon YM-2 membrane, dialyzed against deionized water for 4 hr by using Visking 18 membrane, and lyophilized. After reconstituting in 0.2 M ammonium bicarbonate, the ultrafiltrate was chromatographed successively on Sephadex G-75 superfine, CM-Sephadex C-25, DEAE-Sephadex A-25, Sephadex G-50 superfine, and CM-Sephadex C-25 columns. Experimental details are given under the legends to the individual chromatograms. The compositions of the fractions at each purification step were monitored by agarose gel electrophoresis (18), and by immunoelectrophoresis or fused rocked immunoelectrophoresis (19) by using antisera against LPE, *Lol p I*, *II*, and *III*.

Agarose electrophoresis and immunoelectrophoresis. Agarose electrophoresis was performed at 10°C in 1% agarose (SeaKem ME Agarose, FMC Corporation) with Tris-tricine buffer (pH 8.6), 1.0 l according to Roebber et al. (18). Proteins were run for 45 min at 10 V/cm and then stained with "Boston" stain (50 ml methanol, 124 ml water, 20 gm trichloroacetic acid, 6 g sulfosalicylic acid, 0.18 g Coomassie brilliant blue R250). Immunoelectrophoresis was performed as previously described (18).

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed in slab gels following the method of Laemmli (20). The sample (4 to 5 μ g) and m.w. standards were denatured and reduced by boiling in 62 mM Tris-HCl (pH 6.8), containing 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol, and then electrophoresed for 4 hr at 30 mA in the gel (1.5 mm thick, 12 cm long, 14 cm wide). The stacking gel (4% concentration) was prepared in 125 mM Tris-HCl (pH 6.8) containing 0.1% SDS. The separating gel consisted of a gradient of 10 to 20% gel in 375 mM Tris-HCl (pH 8.8) with 0.1% SDS. The tank buffer was 25 mM Tris-192 mM glycine (pH 8.3) with 0.1% SDS. After the electrophoresis, the proteins were stained in 0.125% (w/v) Coomassie blue R-250 prepared in 50% methanol-10% acetic acid.

Isoelectric focusing. Isoelectric focusing was performed at 10°C with LKB Ampholine PAG plates in the pH range 3.5 to 9.5 following the manufacturer's directions. Standard proteins of known isoelectric point were used as markers (Isogel isoelectric point markers, FMC Corporation). The gels were fixed, and stained with Coomassie brilliant blue R250.

Double Ab radioimmunoassays (DARIA). Each *Lol p* antigen was radiolabeled with ^{125} I by the chloramine T method (21) to a specific activity of 9×10^4 cpm/ng. Direct-binding DARIA was used to determine the concentrations of human IgG Ab to *Lol p I*, *II*, and *III* (8). The minimum detectable level of serum IgG Ab was ca. 0.3 ng in 100 μ l of the human serum used in each assay tube. All determinations were performed in triplicate in at least two assays. Further experiments were performed where the coefficient of variation was >10% for the means of the first two assays. Sometimes as many as four assays were necessary to finalize the data where the Ab levels were low.

Cross-reaction among *Lol p I*, *II*, and *III* was studied at the IgG Ab level by using inhibition DARIA in which the ability of each of the three antigens (unlabeled) to inhibit the binding of radiolabeled *Lol p I*, *II*, or *III* was investigated with human, goat, and rabbit sera.

Appropriately diluted human, goat, or rabbit serum (100 μ l, containing approximately 30 ng specific IgG Ab) was added to a 100- μ l mixture containing 1 ng labeled antigen and varying amounts of unlabeled inhibitor in 5% bovine serum albumin. (In absence of an inhibitor, 30 ng of the specific Ab causes approximately 10% binding of the respective labeled antigen; all the inhibition studies were carried out at this level of antigen binding. We found no difference in the inhibition results when we used serum dilutions containing 150 ng of specific Ab, which causes 27 to 41% binding of the respective antigen in the absence of an inhibitor.) After 4 hr at 23°C, 100 μ l of second Ab (goat anti-human IgG, γ -chain specific, rabbit anti-goat IgG, or goat anti-rabbit IgG) were added. After overnight incubation at 4°C, the resultant precipitates were washed four times and counted in a gamma counter. Percent inhibition was calculated with the equation: % inhibition = $100(C_0 - C_i/C_0)$, where C_0 and C_i are the net cpm in the absence and in the presence of inhibitor, respectively.

RESULTS

Purification of *Lol p III*. Purification of *Lol p I* and *II* has been described earlier (8, 16). Purification of *Lol p III* involved six successive chromatographic steps depicted in Figure 1. The starting material was a "medium m.w. fraction" of the pollen extract. This fraction was obtained by removing high m.w. and low m.w. substances first by passing the pollen extract through an Amicon hollow-fiber H1P5 cartridge and discarding the retentate, and then passing the ultrafiltrate through an Amicon YM-2 membrane followed by dialysis and lyophilization of the YM-2 retentate. On Sephadex G-75 column (Fig. 1A), the "medium m.w. fraction" was resolved into four protein peaks and a fifth, broad band containing predominantly low m.w. pigments (not shown in Fig. 1A).

Lol p III was identified in the fourth peak by agarose electrophoresis and immunoelectrophoresis. The fractions under this peak were pooled, concentrated through an Amicon YM-2 membrane, diluted with water to 25 ml and chromatographed on a CM-Sephadex column (Fig. 1B). Acidic proteins were eluted in two peaks in the initial buffer (0.01 M ammonium bicarbonate, pH 8.0), followed by other proteins when a salt gradient was applied. Peak 4 (Fig. 1B), containing *Lol p III*, was concentrated through a YM-2 membrane and diluted with water to decrease the salt concentration in the protein to less than 0.01 M. It was then chromatographed on a DEAE-Sephadex A-25 column (Fig. 1C). Two peaks were obtained; peak 2 was identified to contain *Lol p III*. On Sephadex G-50 chromatography this protein yielded a single peak, which was further chromatographed twice on CM-Sephadex to obtain a pure preparation of *Lol p III*.

Characterization of the purified *Lol p I*, *II*, and *III*. On agarose electrophoresis the purified *Lol p I*, *II* and *III* preparations each gave single well-defined bands (Fig. 2A). In a separate experiment (not shown), the purified *Lol p I* was identified to be predominantly the isoallergenic form *Lol p IC* (22). The *Lol p II* preparation was tentatively identified to be the form *Lol p IIA*, and the *Lol p III* preparation corresponded to the form *Lol p IIIB*. On SDS-PAGE (Fig. 2B), each of the proteins gave one band corresponding to its reported m.w. (*Lol p I* 32,000, *Lol p II* 11,000, and *Lol p III* 11,000). [It should be noted that *Lol p I* gives different values for its m.w. by different methods: 27,000 by amino acid analysis (23); 31,000 by gel filtration (24); and 32,000 by sedimentation equilibrium (25), and SDS-PAGE (D. G. Marsh, unpublished observation)]. On isoelectric focusing (Fig. 2C) *Lol p I* and *II* each gave a major band with evidence of contamination

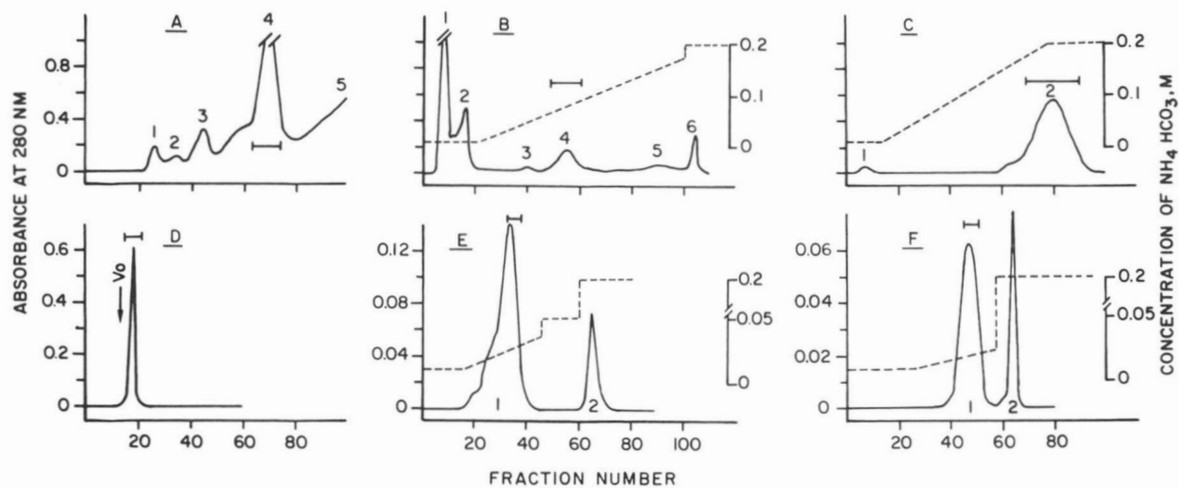
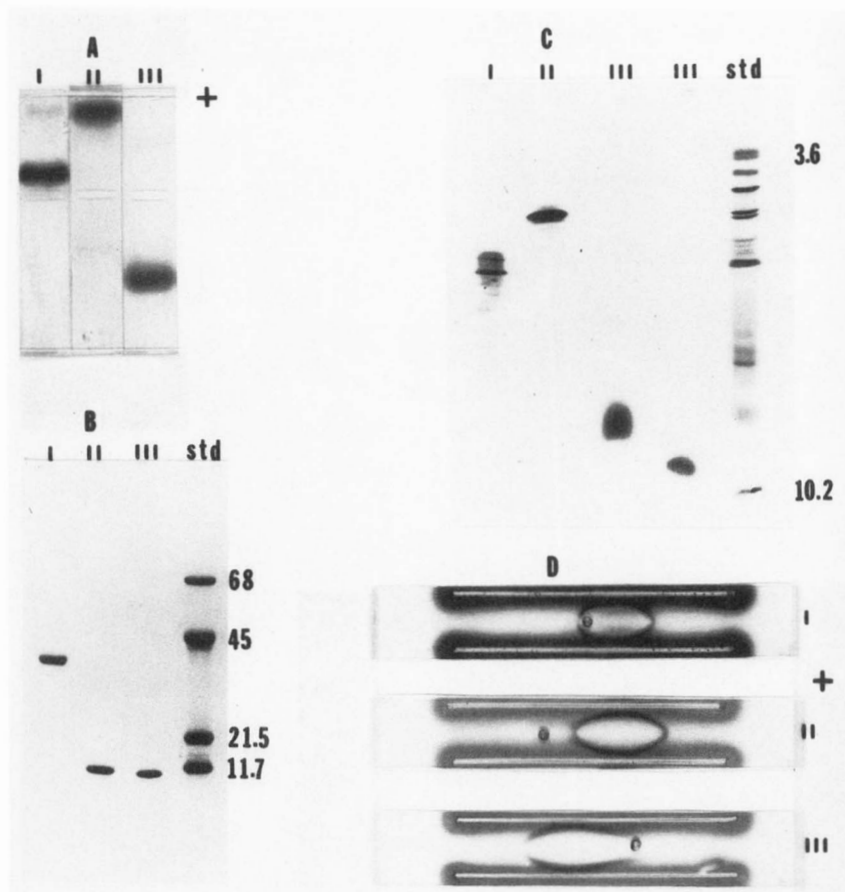


Figure 1. Purification of *Lol p III*. The fractions containing *Lol p III* are indicated by horizontal bars. A, Sephadex G-75 superfine chromatography of rye grass pollen extract. Column: 5 × 95 cm; sample: 15 ml containing 1.28 g of lyophilized material; eluent: 0.2 M ammonium bicarbonate (pH 8.0) containing 0.02% sodium azide; flow rate: 20 ml/hr; fraction size: 10 ml. B, CM-Sephadex C-25 chromatography of *Lol p III*-containing fraction (peak 4 from A). Column: 2.6 × 40 cm; sample: approximately 100 ml of the pool (peak 4, in A) was concentrated to 25 ml through Amicon YM-2 membrane and applied on this column; elution: initially with 0.01 M ammonium bicarbonate, pH 8.0, then linear gradient with increasing ammonium bicarbonate concentration (to 0.1 M) at constant pH of 8.0, finally with 0.2 M ammonium bicarbonate plus ammonium hydroxide (pH 9.1); flow rate: 40 ml/hr; fraction size: 10 ml. C, DEAE-Sephadex A-25 chromatography of peak 4, in B. Column 2.5 × 52 cm; sample: 10 ml; elution: concentration gradient of ammonium bicarbonate plus ammonium hydroxide, pH 9.0; flow rate: 28 ml/hr; fraction size: 15 ml. D, Sephadex G-50 superfine chromatography of peak 2, in C. Column: 1.4 × 160 cm; sample: 3.3 ml; eluent: 0.2 M ammonium bicarbonate (pH 8.0); flow rate: 15 ml/hr; fraction size: 7.5 ml. E, CM-Sephadex C-25 chromatography of the peak from D. Column: 1.4 × 16 cm; sample: 3 ml; elution: concentration gradient of ammonium bicarbonate (pH 8.0); flow rate: 30 ml/hr; fraction size: 4 ml. F, CM-Sephadex C-25 chromatography of peak 1 from E. Column: 1.4 × 16 cm; sample: 2 ml; elution: concentration gradient of ammonium bicarbonate (pH 8.0); flow rate: 30 ml/hr; fraction size: 4 ml.

Figure 2. A, Agarose gel electrophoresis of purified *Lol p I*, II and III at pH 8.6. The electrophoresis was performed at 10°C in 1% agarose (SeaKem ME Agarose, FMC Corporation) using Tris-tricine buffer (pH 8.6), 10.1. Proteins (about 6 µg) were run for 45 min at 10 V/cm and then stained using "Boston" stain. The diagram was assembled from three different runs. B, SDS-PAGE analysis of purified *Lol p I*, II, and III in a 10 to 20% gradient gel. Four- to 5-µg samples were applied. The m.w. markers were from bottom to top: horse heart cytochrome c (11,700), soyabean trypsin inhibitor (21,500), ovalbumin (45,000) and bovine serum albumin (68,000). C, Isoelectric focusing of purified *Lol p I*, II and III (6 to 8 µg) at 10°C with the use of LKB Ampholine PAG plates. The gels were stained with Coomassie brilliant blue R250. D, Immunoelectrophoresis of purified *Lol p I*, II and III using a highly potent antiserum pool (G797-9) from three goats hyperimmunized with crude rye pollen extract. Samples were run at 10°C for 45 min at 10 V/cm in 1% agarose in Tris-tricine buffer (pH = 8.6, I = 0.1), and antiserum was added to troughs cut in the gels, which were then incubated overnight at 23°C. After washing and drying, the gels were stained with 0.1% Coomassie blue R250 in 45% methanol, 10% acetic acid. The diagram is assembled from three different runs.



with its respective isoallergenic forms (22); apparent isoelectric points of the major bands of *Lol p I* and II were estimated to be 5.9 to 6.0 and 5.0 to 5.2, respectively, based on the standard protein markers. Isoelectric focus-

ing of two different preparations of *Lol p III* gave diffuse bands which did not focus properly in repeated experiments; the more basic of the two preparations, *Lol p IIIA* (pI ca. 9.4) was obtained earlier (A. A. Ansari, unpub-

lished results) and the less basic preparation (pI 9.0) was obtained as described in this paper. The major, less basic form, *Lol p IIB*, was used throughout this paper. Immunoelectrophoresis was performed using a pool of antisera from three goats hyperimmunized with crude rye pollen extract. This antiserum pool (also available as an NIH Research Resources Reagent, No. A-617-601-557) gives at least 15 precipitin lines by immunoelectrophoresis against LPE (4). Purified *Lol p II* and *III* gave only one precipitin line with this antiserum (Fig. 2D), whereas *Lol p I* showed evidence of a trace impurity.

Antibody positivity to *Lol p I*, *II*, and *III*. We determined IgG Ab levels against *Lol p I*, *II* and *III* by DARIA in 189 allergic Westinghouse subjects. Table I shows a comparison of the prevalence of Ab levels to pairs of the *Lol p* allergens. Many of these subjects exhibited Ab to one allergen in absence of detectable Ab to other allergen (Table I). Thus, among 128 *Lol p I* IgG Ab+ subjects, 53% did not show detectable Ab to *Lol p II* (Table I, line 1). Obviously, the *Lol p I* IgG Ab of these 53% subjects did not cross-react with *Lol p II*, otherwise some of the anti-*Lol p I* Ab would have been detected as anti-*Lol p II* Ab in the DARIA by using labeled *Lol p II*. The remaining 47% of the *Lol p I* IgG Ab+ subjects were also positive for IgG Ab against *Lol p II*, either because of cross-reactivity between *Lol p I* and *Lol p II* and/or because of the presence of Ab against both the antigens. Similarly, the *Lol p I* IgG Ab of at least 63% of the subjects did not cross-react with *Lol p III* (Table I). No concordance of Ab positivity (hence lack of crossreactivity) was also observed, although in fewer subjects, in the other comparisons shown in Table I.

Inhibition studies using human sera. For the inhibition assays, we used sera from another group of individuals who had been on grass immunotherapy for two or more years. These sera were selected on the basis of their high levels of IgG Ab against two or more of the three allergens. The detection of Ab against more than one of the allergens would be expected either because of cross-reactivity between the allergens and/or because of the presence of non-cross-reacting Ab against the allergens, or from a combination of these two factors. In order to investigate these possibilities, we carried out a series of experiments in which each unlabeled allergen was used to inhibit the binding of the labeled allergens to IgG Ab in each patient's serum.

Figure 3 shows inhibition curves for the binding of

INHIBITION OF *Lol p I* BINDING BY: ■ *Lol p I*
○ *Lol p II*
● *Lol p III*

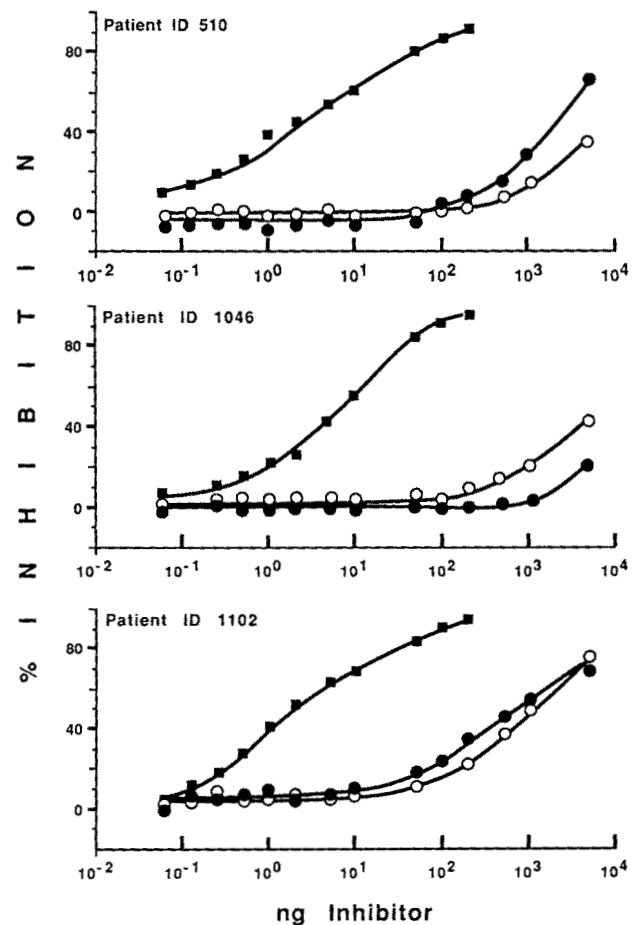


Figure 3. Inhibition by *Lol p I*, *II*, and *III* of the binding of labeled *Lol p I* to three human sera containing IgG Ab to all the three antigens.

radiolabeled *Lol p I* to the IgG Ab in three representative human sera. The homologous unlabeled inhibitor (*Lol p I*) gave approximately 50% inhibition at 1 ng, an amount equivalent to that of the unlabeled antigen in each assay tube. At higher amounts of the inhibitor, the level of inhibition for the homologous inhibitor approached 100%. The heterologous inhibition curves with the use of unlabeled *Lol p II* and *Lol p III* had similar slopes, but were shifted to the right by three to four orders of magnitude. For all the three sera, the *Lol p II* and *Lol p III* heterologous inhibition curves yielded similar K_{rel} values (K_{rel} = amount of heterologous inhibitor required for 50% inhibition divided by the amount of homologous inhibitor required for 50% inhibition). These observations are consistent with the possibility that *Lol p II* and *III* preparations are contaminated with low levels (ca. 0.1 to 0.01%) of *Lol p I*, and the observed heterologous inhibition curves might result from this contamination. It is logical to assume that such trace contamination could go undetected by the usual criteria of purity applied to these proteins, namely, Coomassie blue staining after agarose electrophoresis, SDS-PAGE, isoelectric focusing and immunoelectrophoresis.

Figure 4 shows a set of inhibition curves by using the same three human sera; the labeled antigen in this case was *Lol p II* instead of *Lol p I*. In the case of one serum

TABLE I

Correlations between IgG Ab positivity to pairs of antigens, *Lol p I*, *II*, and *III*^a

Group Description ^b	Proportion (%)
1. II ⁺ I ⁺	68/128 (53)
2. III ⁺ I ⁺	81/128 (63)
3. I ⁺ II ⁺	9/69 (13)
4. III ⁺ II ⁺	17/69 (25)
5. I ⁺ III ⁺	9/56 (16)
6. II ⁺ III ⁺	4/56 (7)

^a This analysis was done on a total population of 189 subjects, including 122 subjects who were ST⁺ to LPE, and 67 subjects who were ST⁻ to LPE but ST⁺ to one or more of other inhalant allergens (extracts of short ragweed and timothy grass pollen, cat dander, house dust, and *Alternaria* spp.).

^b The word *Lol p* has been eliminated from the group description for the sake of simplicity. Thus, the group II⁺ I⁺ is comprised of subjects who were Ab⁺ to *Lol p I* but not to *Lol p II*. For example, there were 128 subjects who had IgG Ab to *Lol p I*, out of which 68 subjects (53%) had no detectable IgG Ab to *Lol p II* (see line 1).

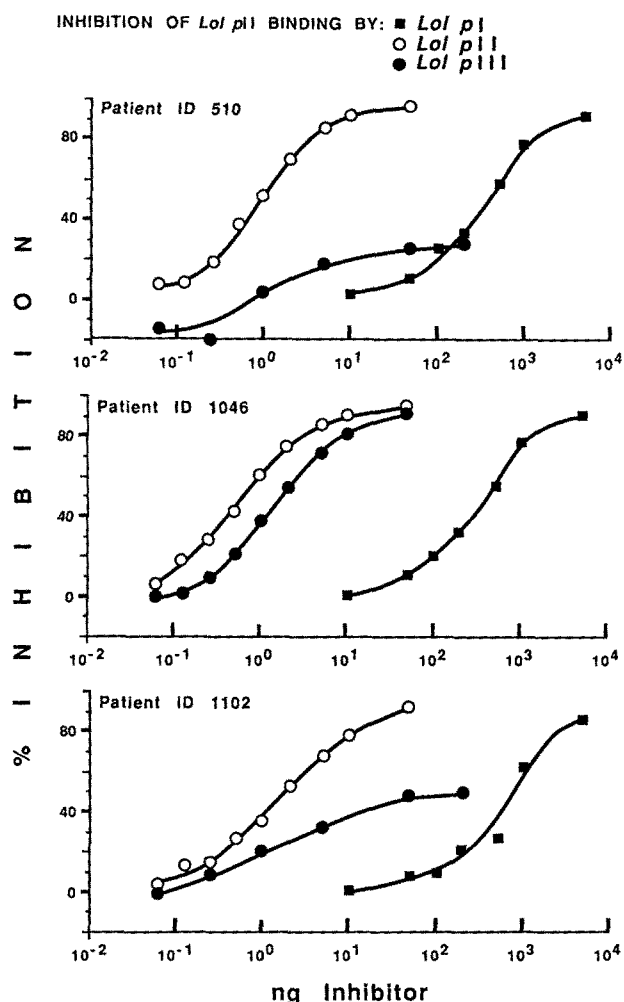


Figure 4. Inhibition by *Lol p I*, II, and III of the binding of labeled *Lol p I* to three human sera containing IgG Ab to all the three antigens.

(No. 1046) the shape of the inhibition curve for the heterologous antigen, *Lol p III* closely follows that for the homologous antigen, *Lol p II*, although it is shifted to the right by approximately 2-fold (perhaps resulting from experimental error; see below). This result indicates a high degree of cross-reactivity between *Lol p III* and *Lol p II* in the case of this patient's IgG Ab. For the other two sera, the slopes of the *Lol p III* inhibition curves are clearly different from those of the *Lol p II* inhibition curves, and indicate much lower cross-reactivity between *Lol p III* and *Lol p II*. For all three sera, the *Lol p I* inhibition curves are consistent with ca. 0.2% contamination by *Lol p II* in the *Lol p I* preparation.

Figure 5 shows the analogous set of inhibition curves for labeled *Lol p III*. For serum 1046, the magnitude of the heterologous *Lol p II* inhibition is slightly greater than that of the homologous inhibition, but this difference is within the experimental error of ca. 10%. The binding of labeled *Lol p III* to IgG Ab in all three sera is strongly inhibited by *Lol p II*, indicating a high degree of cross-reactivity between *Lol p II* and *Lol p III*. As in the previous two sets of inhibition data (Figs. 3 and 4), the *Lol p I* inhibition curves in Figure 5 are consistent with trace contamination (0.1 to 0.2%) by *Lol p II* and/or III in the *Lol p I* preparation.

From these data it appears that the observed cross-

inhibition between *Lol p II* and III reflect partial or complete cross-reactivity, but the observed cross-inhibition of *Lol p I* with *Lol p II* or III might result from low-level cross-contamination between the three proteins, which is undetectable by conventional physicochemical and immunologic analyses. In order to check this possibility, we used three further human sera that had high levels of IgG Ab against *Lol p I* but no detectable Ab to *Lol p II* and III, and, therefore, contained no cross-reacting Ab. In the inhibition studies using these sera, both the *Lol p II* and III preparations were able to inhibit the binding of labeled *Lol p I* (Fig. 6A). The heterologous inhibition curves obtained for *Lol p II* and III were parallel to the homologous inhibition curves for *Lol p I* in all the three sera. For all the three sera the *Lol p II* and III curves were respectively displaced to the right by the same magnitude in relation to the homologous *Lol p I* inhibition curves; the K_{rel} values for *Lol p II* and III were close to 1300 and 4100, respectively, in each of the three sera. Since IgG Ab in these sera do not bind *Lol p II* and III, we conclude from these inhibition results that the *Lol p II* preparation contains *Lol p I* to a level of about 1 in 1300 (0.08%), and the *Lol p III* preparation contains *Lol p I* to a level of 1 in 4100 (0.02%).

Evidence of cross-contamination between *Lol p II* and III was obtained with three additional human sera that

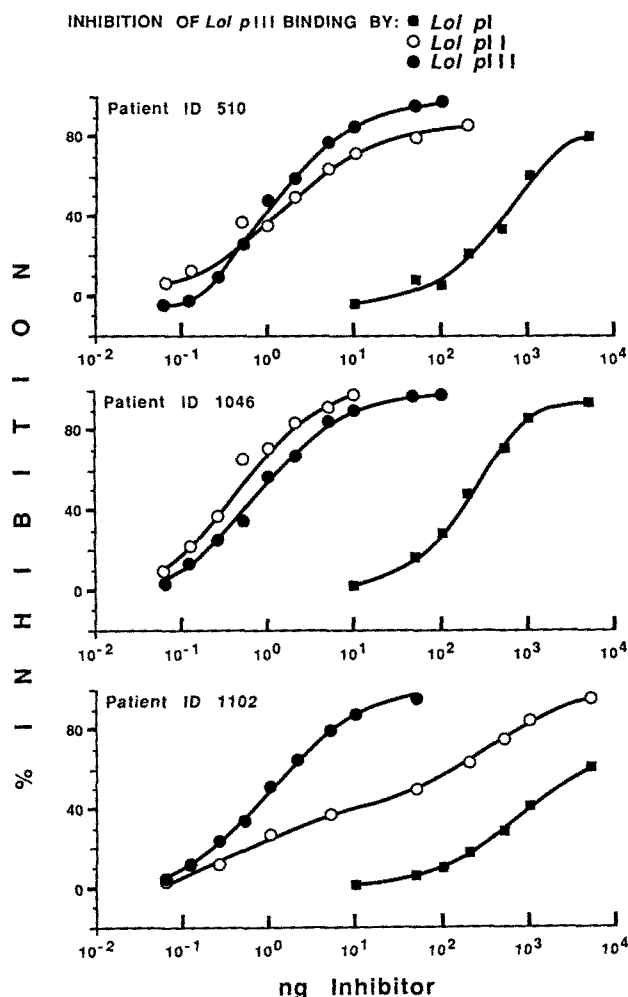


Figure 5. Inhibition by *Lol p I*, II, and III of the binding of labeled *Lol p III* to three human sera containing IgG Ab to all the three antigens.

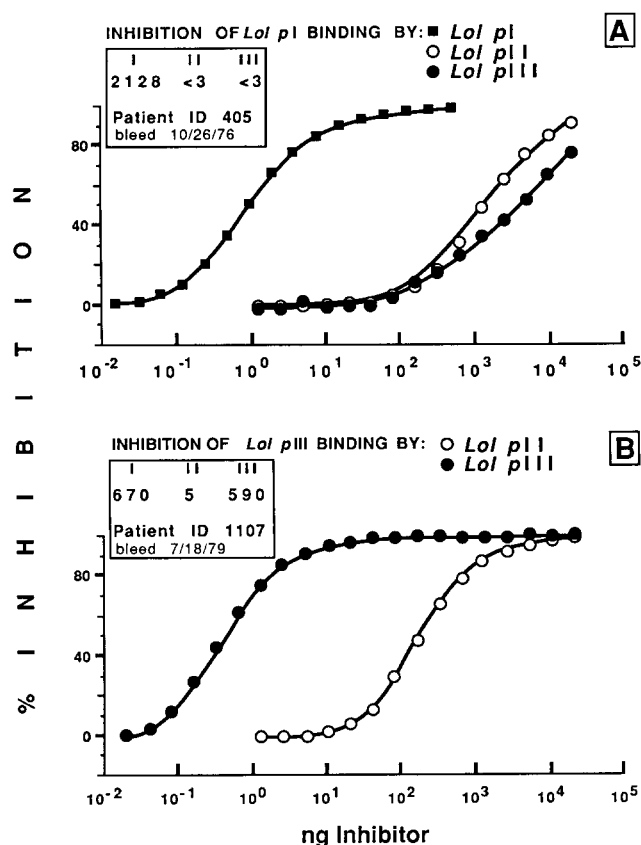


Figure 6. A, Inhibition by *Lol p I*, II, and III of the binding of labeled *Lol p I* to a human serum containing IgG Ab to only *Lol p I*. The numbers below I, II, and III are specific IgG Ab levels (ng/ml) for *Lol p I*, II and III, respectively. B, Inhibition by *Lol p II* and III of the binding of labeled *Lol p III* to a human serum containing little or no IgG Ab to *Lol p II*. The numbers below I, II, and III are specific IgG Ab levels (ng/ml) for *Lol p I*, II, and III, respectively.

had high levels IgG Ab against *Lol p III* but little or no detectable Ab against *Lol p II*. Representative inhibition curves with the use of one of the three sera are shown in Figure 6B. From these results, we conclude that the *Lol p II* preparation contains a low level of *Lol p III* (ca. 0.1%), undetectable by the other techniques.

In order to avoid interference from low-level cross-contamination, further inhibition studies were carried out with 10 ng of the inhibitors (10-fold excess over the labeled antigen). As can be seen from Figures 3 to 6, the contribution of the contaminants to the percent inhibition values would be insignificant at the level of 10 ng of inhibitor. Figure 7 shows such inhibition data on 13 human sera including those presented in Figures 3 to 5 (sera 405 and 1103 were not run with labeled *Lol p III* because of their low anti-*Lol p III* Ab content). Most of the sera show appreciable degrees of two-way cross-reactivity between *Lol p II* and III and no significant cross-reactivity between *Lol p I* and II or between *Lol p I* and III.

Cross-reaction with the use of animal sera. Inhibition studies were also performed with sera from one goat and six rabbits immunized with either LPE or partially purified *Lol p I* or *Lol p II* (Fig. 8). All these antisera had Ab against *Lol p I*, II, and III, except for two rabbit sera, R107 and R138, which had low Ab levels against *Lol p II* and III, and therefore were not run in the inhibition assays with labeled *Lol p II* and III. As in the case of human sera,

the binding of *Lol p I* to any of the animal sera was not inhibited by *Lol p II* or III; nor did *Lol p I* inhibit the binding of *Lol p II* or III to the animal sera. These results indicate a lack of detectable cross-reactivity between *Lol p I* and II or between *Lol p I* and III. The binding of *Lol p II* to the goat serum, but not to the four rabbit sera used, was inhibited by *Lol p III*. The binding of *Lol p III* to three rabbit sera, but not to the goat serum and the fourth rabbit serum, was inhibited by *Lol p II*. These results indicate a two-way cross-reactivity between *Lol p II* and III. Thus, the results obtained from animal sera were similar to those obtained from human sera, although the degree of cross-reactivity detected by the animal sera was lower.

DISCUSSION

We purified *Lol p I*, II, and III to virtual homogeneity. No impurities were detected in any of the three antigens by Coomassie blue staining after agarose gel electrophoresis, SDS-PAGE, and isoelectric focusing; only *Lol p I*

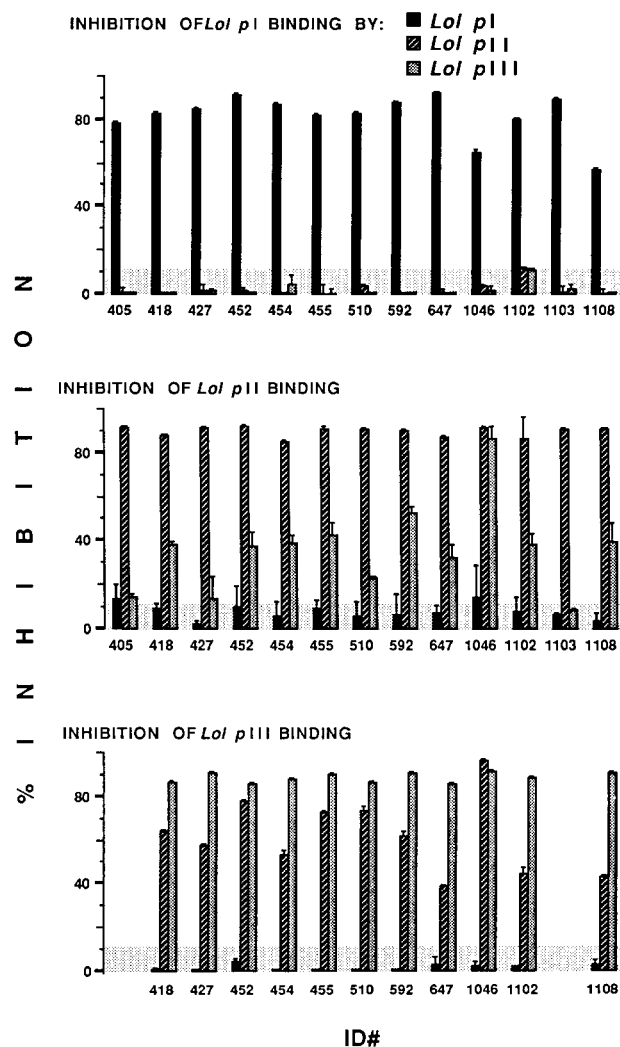


Figure 7. Cross-reactivities between *Lol p I*, II, and III exhibited by IgG Ab of 13 human sera containing Ab to all the three antigens. Data for 10 ng of homologous and heterologous inhibitors, representing a 10-fold weight excess over the labeled antigen, are shown. The labeled antigens were: *Lol p I* (top), *Lol p II* (center), and *Lol p III* (bottom). Sera 405 and 1103 were not run with labeled *Lol p III* because their anti-*Lol p III* Ab contents were too low. The horizontal hatched area shows insignificant level of inhibition.

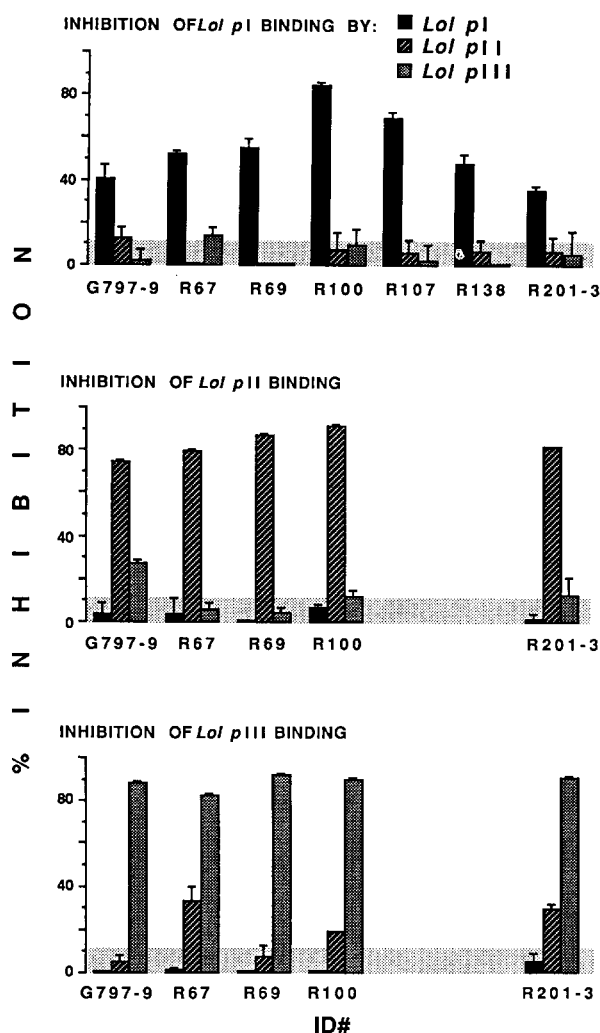


Figure 8. Cross-reactivities between *Lol p I*, II and III exhibited by IgG Ab of goat (G) and rabbit (R) antisera containing Ab to all the three antigens. Data for 10 ng of homologous and heterologous inhibitors, representing a 10-fold weight excess over the labeled antigen, are shown. The labeled antigens were: *Lol p I* (top), *Lol p II* (center), and *Lol p III* (bottom). The horizontal hatched area shows insignificant level of inhibition. Although the inhibition of *Lol p II* and III binding (center and bottom panels) was close to 80% with 10 ng of the respective homologous inhibitors, the level of homologous inhibition obtained for *Lol p I* binding to various animal sera ranged from 40 to 82% (top panel). Similar results were obtained in three experiments, including analysis of complete inhibition curves, similar to those in Figures 3 to 6. The reason for this discrepancy seen only with *Lol p I* is not clear.

showed a trace of impurity in immunoelectrophoresis. Although isoelectric focusing did show the presence of a few minor bands in *Lol p I* and II preparations, these extra bands most probably represent isoallergenic forms (22) of the respective proteins, inasmuch as they are immunologically indistinguishable from the respective major component in immunoelectrophoresis (Fig. 2). *Lol p I* is known to exist in at least four isoallergenic forms, I-A, I-B, I-C, and I-D, whereas *Lol p II* and III each exists in two isoallergenic forms, A and B. In each case, these isoallergenic forms are immunologically indistinguishable (22) but differ slightly in their isoelectric points. It should be noted that none of the above-mentioned four techniques showed any evidence of cross-contamination of the three proteins. However, our inhibition DARIA data (Figs. 3 to 6) demonstrated that the three protein preparations were cross-contaminated with each other at levels

(0.01 to 0.2%) that would go undetected in the other techniques we used to check the purity of the protein preparations.

Evidence for the presence of low-level contamination is derived from the data in Figures 3 to 6 (see Results). The estimated 0.2% contamination of *Lol p I* with *Lol p II*, for example, would present no complication in direct binding DARIA with the labeled *Lol p I* preparation, inasmuch as it would increase the bound cpm by only 0.2%. However, such contamination becomes apparent in inhibition DARIA when ratios of 1000:1 of heterologous inhibitor to radiolabeled antigen are used. In order to avoid the interference from cross-contamination in our inhibition studies, we used only a 10-fold excess of the unlabeled inhibitors over the labeled antigen in each assay tube (Fig. 7).

The cross-reactivity data can be summarized as follows. The binding of labeled *Lol p I* to its IgG Ab is not inhibited by *Lol p II* or III (Figs. 3 and 7, top panel), nor does *Lol p I* inhibit the binding of labeled *Lol p II* or III to their respective IgG Ab (Figs. 4, 5, and 7, center and bottom panels); thus, there is no appreciable cross-reactivity between *Lol p I* and II or between *Lol p I* and III. However, there is clear evidence for two-way cross-reactivity between *Lol p II* and III for certain sera; the binding of *Lol p II* is appreciably inhibited by *Lol p III* (Figs. 4 and 7, center panel), and vice versa (Figs. 5 and 7, bottom panel), degree of cross-inhibition varying between undetectable and essentially complete. It should, however, be noted that the sera were selected on the basis of their high antigen-binding values for at least two antigens in order to maximize the chance of detection cross-reaction, if any. There are 25% subjects who have anti-*Lol p II* IgG Ab that show no measurable binding to *Lol p III* (Table I, line 4), and 7% who have anti-*Lol p III* IgG Ab that do not cross-react with *Lol p II* (Table I, line 6); in such cases there is clearly no detectable cross-reactivity.

With goat and rabbit antisera, no cross-reactivity was found between *Lol p I* and the other two antigens, and the degree of cross-reactivity between *Lol p II* and III was less than that observed by using human sera. Like many human sera, some rabbit antisera did not detect any cross-reactivity between the three antigens, which is consistent with an earlier observation in our laboratory (10) and a preliminary observation by Hull et al. (26).

From these studies we conclude that: 1) *Lol p I* is virtually non-cross-reactive with *Lol p II* and III; 2) *Lol p II* and III are cross-reactive with some human and animal sera; and 3) different human and animal sera exhibit varying degrees of cross-reactivities—different individual's IgG Ab "see" different antigenic determinants (B cell determinants). For those sera that show Ab levels against both *Lol p II* and III, it has not been possible to determine the contribution of Ab against one antigen in the measurement of Ab against the other by the DARIA.

In a separate set of studies (8, 9), we have found that IgG and IgE Ab responses to *Lol p I*, II, and III are associated with HLA-DR3, and that most subjects who respond to *Lol p II* and III also respond to *Lol p I*, but all *Lol p I* responders do not necessarily respond to *Lol p II* and III. From our inhibition studies, it now becomes clear that the concordance of Ab to *Lol p I* and II, or *Lol p I* and III must be the result of a subject's specific immune response to each of these antigens, and not the result of

cross-reactivity between them. However, since *Lol p* II and III show cross-reactivity with IgG Ab of some subjects, a similar conclusion can not be drawn about the concordant responsiveness to *Lol p* II and III. Taken together these findings suggest that, while *Lol p* I does not share any B cell determinant with *Lol p* II or III, probably a similar, immunodominant Ia recognition site is present in *Lol p* I and *Lol p* II and/or III. We postulate that this site interacts with the Ia molecule encoded by the *DR3* gene. In addition, *Lol p* I appears to contain unique Ia recognition site(s) not shared with *Lol p* II and III. These postulates explain why Ab responses to the three allergens are associated with *DR3*, and why most *Lol p* II and III responders are also *Lol p* I responders, but the *Lol p* I responders are not necessarily *Lol p* II and III responders.

On this basis, we expect to find some amino acid sequence homology between these antigens, especially between *Lol p* II and III. The existence of structural homology between *Lol p* I on the one hand and *Lol p* II and III on the other should be useful in helping to delineate possible sites of interaction with HLA-*DR3*. Such structural studies should also be of great help in defining the B cell and T cell sites of the three antigens. We believe that such studies of the molecular basis of the genetic control of human immune responsiveness to these three antigens will also lead to a better understanding of the HLA-*DR3*-associated immune disorders (e.g., myasthenia gravis, systemic lupus erythematosus, primary Sjögren's syndrome, and insulin-dependent diabetes), because similar epitopes might be involved in these disorders and in Ab responses to the *Lol p* allergens.

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