

Biochemical and immunological characterization of recombinant allergen Lol p 1

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Pollen from perennial rye grass (*Lolium perenne*), a major cause of type-I allergy worldwide, contains a complex mixture of allergenic proteins among which Lol p 1 is one of the most important. We describe the expression, purification and characterization of a recombinant Lol p 1 overproduced in *Escherichia coli*. The recombinant allergen, expressed in high yields and purified in milligram amounts, bound to specific IgE antibodies from human sera, induced histamine release from sensitized human basophils, and elicited rabbit antisera that recognize specifically recombinant Lol p 1 and natural Lol p 1 of pollen extract. Recombinant Lol p 1 was used to develop ImmunoCAP assays for analysis of 150 sera that were Radioallergosorbent test positive to *L. perenne* pollen. In 130 of them (87%) the assay detected a significant level of IgE antibodies to Lol p 1, reaching on average 37% of the level obtained with a test for IgE to the whole grass pollen extract. To map epitopes on Lol p 1, we produced three deletion mutants [des-(116–240)-Lol p 1, des-(1–88)-Lol p 1 and des-(133–189)-Lol p 1], which were efficiently expressed in bacteria. These all showed a strong reactivity with the specific rabbit IgG antibodies, but lacked most or all the allergenic properties of recombinant Lol p 1. A study of the antigenic structure of Lol p 1 was performed using the three deletion mutants and a set of 17–18-residue overlapping synthetic peptides covering the whole allergen sequence. The results indicate that human IgE and rabbit IgG antibodies bind to distinct regions of Lol p 1, and that at least some important IgE epitopes are mainly conformational. The findings suggest that recombinant allergens constitute useful reagents for further development of serological diagnosis of allergy, and that it should be possible to produce immunogenic fragments of allergenic proteins without allergenic properties.

Keywords: perennial rye grass; *Lolium perenne*; recombinant allergen; recombinant Lol p 1; allergen mutant.

Grass-pollen allergy occurs world wide, affecting about 75% of all allergic subjects (Freidhoff et al., 1986). The perennial rye grass (*Lolium perenne*) is considered the major source of allergenic pollen (Wutrich, 1989) due to its widespread distribution and its abundant production of pollen during flowering (Smart et al., 1979). Its pollen contains at least 17 allergens ranging from 12 kDa to 89 kDa (Ford and Baldo, 1986), which have been classified on the basis of their electrophoretic mobilities. The major allergens identified belong to groups 1, 2, 3 and 5, and are present in various isoforms. These proteins have been purified and analysed in some detail, and some of them have been produced as recombinant, non-fusion allergens. Among these, a protein of group 2 (Lol p 2), to which about 45% of rye-grass-allergic patients are reactive (Freidhoff et al., 1986), was expressed in *Escherichia coli* in high yields (Sidoli et al., 1993; Tamborini et al., 1995) and shown to have immunological properties analogous to the natural allergen. Recombinant Lol p 2

was used to select recombinant antibody fragments (Fab) from a combinatorial library of human antibody cDNA sequences, which could inhibit the triggering of histamine release by Lol p 2. The recombinant allergen was used to map epitopes for IgE and IgG (de Lalla et al., 1996).

Earlier studies showed that group-1 allergens comprise glycosylated proteins with apparent molecular masses of 28–35 kDa (Freidhoff et al., 1986) that are major IgE-binding components (Marsh et al., 1966). Elevated levels of IgE antibodies to group-1 allergens of *L. perenne* were found in sera of 95% of subjects allergic to rye grass (Kahn and Marsh, 1986). It was later shown that the group comprises two major allergens: Lol p 1 of 35 kDa (Perez et al., 1990; Griffith et al., 1991), previously referred as Lol p 1a; and Lol p 5 of 31 kDa (Ong et al., 1993), previously referred to as Lol p 1b (Singh et al., 1991) or Lol p 9 (Suphioglu et al., 1992). Lol p 1 and Lol p 5 have no significant amino acid sequence similarity, and differ in cellular localization: Lol p 1 is deposited on the surface of the pollen; while Lol p 5 is present in amyloplasts (Suphioglu et al., 1992; Taylor et al., 1994). Purified Lol p 1 and Lol p 5 inhibited almost completely serum IgE binding to rye-grass pollen extracts (Bond et al., 1993). Lol p 5 was recently expressed in *E. coli* as a fusion protein, and its properties appeared similar to those of the natural allergen (Ong et al., 1995).

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Abbreviations. Lol p 1, Lol p 2, Lol p 3, Lol p 5, allergens from perennial rye grass (*Lolium perenne*) belonging to groups 1, 2, 3 and 5, respectively, named according to the WHO/IUIS Allergen Nomenclature; HRP, horseradish peroxidase.

cDNA cloning and sequencing confirmed that Lol p 1 exists in multiple isoforms of the same size, with polymorphisms appearing in at least eight positions in the amino acid sequence (Perez et al., 1990; Griffith et al., 1991). The natural protein has been characterized in some detail and found to be a glycosylated monomer of 35 kDa containing intramolecular disulfide bridges (Singh et al., 1991; Johnson and Marsh, 1965). An IgE-binding region spanning the 29 C-terminal amino acids of the allergen was identified by protein fragmentation (Esch and Klapper 1989a, b), and IgG antibodies directed to a synthetic peptide of the same C-terminal region inhibited Lol p 1-mediated histamine release from basophils of a sensitized subject (van Ree and Aalberse, 1995).

Access to homogenous and abundant protein preparations can facilitate further characterization of the antigenic determinants. For this purpose we have prepared a cDNA for Lol p 1 from pollen mRNA, cloned it into an expression vector, and produced the allergen as a non-fusion protein. The protein was recognized by serum IgE, induced histamine release from sensitized basophils, and was used to develop an ImmunoCAP assay to determine the level of IgE specific for recombinant Lol p 1 in sera from 150 grass pollen allergic subjects. In addition, we expressed three deletion mutants lacking large portions of the protein at the terminal regions and internally. Their characterization, together with studies using overlapping synthetic peptides covering the whole sequence, made it possible to map major epitopes for human IgE and rabbit IgG antibodies and to show that they are physically separated. The finding that two of the mutants did not elicit histamine release from sensitized basophils, although they were recognized by rabbit IgG antibodies, encourages further studies of strategies for specific immunotherapy.

MATERIALS AND METHODS

Materials. Biochemical and immunological reagents were purchased from BDH and Sigma. DNA restriction and modification enzymes were from New England Biolabs, except benzonase, which was purchased from Merck. Synthetic oligonucleotides were provided by Primm.

Expression vectors for Lol p 1 and its derivatives. The cDNA coding for Lol p 1 was obtained by reverse-transcription/PCR, using total RNA prepared from pollen of *L. perenne* (Allergon AB), as described previously (Sidoli et al., 1993). First-strand cDNA was synthesized using avian myeloblastoma virus reverse transcriptase (Invitrogen) following the supplier's recommendations. cDNA encoding Lol p 1 was amplified by a PCR using the following primers, which were derived from the previously reported nucleotide sequence (Perez et al., 1990): 5'-ACTACATATGATTGCTAAGGTTCCGCCGGGTCCGAACAT-TACTGCAGAGTACGGGGAC-3' (forward; *NdeI* site underlined) and 5'-ACTTGGATCCTCATTCAGATCCTTCTG-AGATGAGTTTTTGTCTCTTAGCAGAATAAGACGTGTC-GGCTTTCCAGCC-3' (reverse; *BamHI* site underlined).

After PCR, a single band of the expected size was obtained, which after digestion with *NdeI* and *BamHI* was subcloned into a pET11 vector (Novagen), cleaved with the same restriction enzymes. One clone was characterized by DNA sequencing using an automatic sequencer (373A, Perkin-Elmer) and named pETLol p 1.

From plasmid pETLol p 1, a subfragment was generated by PCR using the primer 5'-ACTACATATGACCGTCACAAT-CACCGACGAC 3' (forward; *NdeI* site underlined), and, as a reverse primer, the one described above.

The amplified fragment, which corresponds to the coding sequence of residues 89–240 of the allergen, was digested with

NdeI and *NcoI* (present in the coding region). The 84-bp *NdeI*–*NcoI* fragment obtained was purified and cloned into pETLol p 1 digested with the same enzymes. The insert of the resulting plasmid, named pETdes-(1–88)-Lol p 1, encodes a protein [des-(1–88)-Lol p 1] corresponding to amino acid residues 89 (Thr) through 240 (Lys) of Lol p 1. To obtain a C-terminal deletion, pETLol p 1 was digested with *NcoI* and *BamHI* and the ends filled-in with Klenow. This plasmid was purified from a 1% agarose gel and circularized. The resulting construct was named pETdes-(116–240)-Lol p 1 and codes for residues 1 (Ile) to 115 (Met) of Lol p 1, plus a C-terminal extension (DPAANKAR-KEAELAAATAEQ) due to the cloning procedures [des-(116–240)-Lol p 1]. To create a Lol p 1 mutant with central portion of the protein deleted, an internal *SacI* fragment was excised from the cDNA insert of pETLol p 1, and the plasmid purified from a 1% agarose gel and circularized. The resulting construct was named pETdes-(133–189)-Lol p 1 and codes for a Lol p 1 polypeptide with a deletion spanning a central domain between residue 133 (Gln) and 189 (Leu) [des-(133–189)-Lol p 1].

Expression and purification of the recombinant allergens. *E. coli* strain BL21(DE3) was transformed with the different constructs. Expression of the recombinant proteins was induced by addition of isopropylthio- β -galactopyranoside to 0.5 mM during the exponential phase of growth at 37°C ($A_{600} = 0.8$) in a 4-l fermentor, as described previously (Tamborini et al., 1995). After 3 h induction, cells were recovered by centrifugation and suspended in 50 mM Tris/HCl pH 8, 1 mM EDTA, 0.1% NaN_3 , 1 mM phenylmethylsulfonyl fluoride, 10 mM leupeptin, 10 mM pepstatin A and 0.01 mM aprotinin. After sonication and clarification, the pellet from the recombinant-Lol-p-1-expressing culture was suspended in 50 mM Tris/HCl pH 8, 0.1% Triton X-100, 2 mM EDTA. The washed pellet was solubilized in 25 mM Tris/HCl pH 7.4, 6 M guanidine/HCl and extensively dialysed against NaCl/P_i (138 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate, pH 7.4).

Processing of the three mutants, which were all expressed in a soluble form, entailed the following modifications to the protocol. After sonication, the lysate was digested with benzonase (Merck), following the manufacturer's recommendations, to reduce viscosity. The clarified supernatant was heated to 70°C for 10 min, centrifuged at 4°C for 20 min at 12000 $\times g$, and the soluble fraction was stored at –20°C. *L. perenne* pollen extract was prepared as described previously (Ansari et al., 1989).

Characterization of the recombinant allergens. Purified recombinant Lol p 1 was submitted to electrospray-mass-spectrometry analysis using a VG BIO-Q triple quadrupole mass spectrometer (VG Biotech). Calibration was performed by separate injections of appropriate proteins with well-defined masses. *L. perenne* pollen extract, recombinant Lol p 1, or the mutants were subjected to SDS/PAGE in 12.5% polyacrylamide gels under reducing conditions. Western blotting was performed, and the nitrocellulose filter was saturated in 20% defatted milk in NaCl/Tris (138 mM NaCl, 25 mM Tris/HCl pH 7.4) for 45 min at 40°C. Subsequent incubation was performed with human serum from grass-pollen-allergic subjects. Immunoreactivity was visualized using horseradish peroxidase (HRP)-conjugated rabbit anti-(human-IgE) IgG (Sigma) diluted 1:250 in 2% defatted milk/NaCl/Tris, and developed by enhanced chemiluminescence (Amersham).

ELISA. Microtiter-plate wells (Nunc) were saturated overnight at 4°C with 10 $\mu\text{g/ml}$ (100 $\mu\text{l/well}$) recombinant Lol p 1 or its mutants [des-(116–240)-Lol p 1, des-(1–88)-Lol p 1 and des-(133–189)-Lol p 1] in NaCl/P_i. Blocking was performed for 2 h at room temperature with 10% defatted milk in NaCl/P_i. Plates were washed in NaCl/P_i containing 0.05% Tween 20 (Sigma). The plates were incubated for 90 min at room temper-

ature with serial dilutions of patient sera or rabbit antiserum at a 1: 500 dilution, all in 2% defatted milk in NaCl/P_i. The final incubation was performed for 1 h at room temperature with HRP-conjugated rabbit anti-IgE antibody (Sigma) diluted 1:500 in NaCl/P_i containing 2% defatted milk, or with HRP-conjugated goat anti-rabbit IgG diluted 1:1000. The colorimetric reaction was performed by adding *o*-phenylenediamine dihydrochloride (Sigma). The absorbance at 492 nm was measured using an ELISA reader.

Preparation and use of recombinant Lol p1 ImmunoCAP assays. ImmunoCAP assays specific for recombinant Lol p1, to be used in the Pharmacia CAP system, were prepared by covalent immobilization of the purified allergen on activated cellulose (Axen et al., 1988). Coupling conditions were optimized to achieve an adequate linear measuring range and a background for negative sera below the conventional cut-off value. All tests were run as fluorescent enzyme immunoassays in duplicates on a AutoCAP instrument, using reagents and procedures as recommended by the system supplier (Pharmacia Diagnostics). Statistical parameters attesting the quality of the assays were calculated using CAP System software. For the study using recombinant Lol p1 immunoassays, sera of 150 subjects were selected from an in-house collection at Pharmacia Diagnostics, on the basis of positive radioallergosorbent tests to *L. perenne* pollen (code g5) as the sole selection criterion. The sera ranged in their g5 values from 0.85 kU/l to above the upper cut-off value of 100 kU/l, with an average of 30.4 kU/l.

Peptide mapping. 22 overlapping peptides spanning the whole recombinant Lol p1 sequence were synthesized as described by de Lalla et al. (1996). Peptide sequences were as follows: a1 (residues 1–17), IAKVPPGNITAEYGDK; a2 (10–27), ITAEYGDKWLDAKSTWYG; a3 (21–38), AKSTWYGKPTGAGPKDNG; a4 (31–48), GAGPKDNGGACGYKDVK; b1 (42–58), GYKDVKAPFNGMTGCG; b2 (53–70), GMTGCGNTPIFKDGRGCG; b3 (63–80), FKDGRGCGSCFEIKCTKP; b4 (70–88), GSCFEIKCTKPESCSGEAV; c1 (78–95), TKPESCSGEAVTVTITDD; c2 (85–103), GEAVTVTITDDNNEPIAPY; c3 (95–113), DNEPIAPYHFDLSGHAFG; c4 (111–128), AFGSMAKKGEEQNVRSA; d1 (127–144), AGELELQFRRVKCKYPD; d2 (134–151), FRRVKCKYPDGTKPTFHV; d3 (144–161), GTKPTFHVEKGSNPNYLA; d4 (157–174), PNYLAILVKYVDGDGDVV; e1 (169–186), GDGDVVAVDIKEKGKDKW; e2 (180–197), EKGKDKWIELKESWGA; e3 (190–207), KESWGA VWRIDTPDKLTG; e4 (203–220), DKLTGPFTVRYTTEGGTK; f1 (213–230), YTTEGGTKSEVEDVIPEG; and f2 (223–240), VEDVIPEGWKADTSYSAK.

The synthetic peptides were used in competition ELISA with rabbit IgG or human IgE to map epitopes on Lol p1, as described previously (de Lalla et al., 1996). Duplicates of antibody samples were mixed with each synthetic peptide at increasing concentrations (0.01–250 µg/ml), and immediately added to microtiter-plate wells previously coated with 10 µg/ml recombinant Lol p1. For mapping of epitopes for human IgE, sera of three allergic patients (E. T., A. B., D. G., class 6 to *L. perenne*) were used at dilutions of 1:2, and the binding was detected by HRP-conjugated rabbit anti-(human IgE) IgG (Sigma). For mapping of epitopes for rabbit IgG, sera of two immunized animals were used at 1:4000 dilutions, and binding was detected with a HRP-conjugated goat anti-rabbit IgG (Sigma). All serum dilutions and reactions were performed in 2% defatted milk in NaCl/P_i, and the wells were washed with 0.05% Tween 20 (Sigma) in NaCl/P_i. The reactions were developed and measured as described above. The same peptides were used in a direct binding assay to analyse rabbit IgG reactivity. ELISA plates (MicroTest III flexible assay plates; Falcon) were coated overnight at 4°C

with the different peptides at 20 µg/ml (50 µl/well) in NaCl/P_i. The wells were blocked for 2 h at 37°C using 3% BSA in NaCl/P_i. A 1:500 dilution of the rabbit anti-serum in NaCl/P_i containing 0.5% BSA, 2.5% normal goat serum and 0.05% Tween 20 was added to the wells and left for 1 h at room temperature. Bound IgG was detected as described above.

Production of polyclonal antiserum in rabbit. Two New Zealand rabbits (Primm) were immunized with 700 µg each purified recombinant Lol p1, divided into four doses, by subcutaneous injections at weeks 0, 3, 4 and 5. The first antigen dose was in complete Freund's adjuvant, and the boosters in incomplete Freund's adjuvant. The titre of the sera was evaluated in ELISA with a Lol p1 solid phase, as described previously (de Lalla et al., 1996).

In vitro histamine release. The test for histamine release in whole blood was performed using a commercial enzyme-immunoassay kit, following the manufacturer's instructions (Immunotech International). Blood samples from patient E. T. (class 6 to *L. perenne*) and a non-allergic subject were collected in heparinized tubes and analysed within 2–3 h. The samples were challenged with protein samples at different concentrations for 30 min at 37°C. After incubation, the amount of released histamine was determined.

RESULTS

Lol p1 expression constructs. cDNA coding for Lol p1 was obtained by reverse-transcription/PCR from mRNA of *L. perenne* pollen with primers designed from the nucleotide sequences reported previously (Perez et al., 1990; Griffith et al., 1991). The cDNA was cloned into the pET11 vector and expressed as a non-fusion protein in *E. coli*. The amino acid sequence of the encoded protein was identical to that reported (Perez et al., 1990), except for the replacements Ala177→Gly and Phe232→Ser (numbering of the precursor protein), which were the result of C→G and T→G substitutions, respectively, in the nucleotide sequence. The first substitution has already been reported (SwissProt accession number P14946), while the second is probably an isoform of Lol p1. In addition, three mutants of Lol p1 were produced using the same expression system. They carried deletions of the first 88 N-terminal residues [des-(1–88)-Lol p1], the last 125 C-terminal residues [des-(116–240)-Lol p1], and a central part of 57 residues from position 133 to 189 [des-(133–189)-Lol p1], with residue numbering of the mature protein as in (Perez et al., 1990). A representation of the mutants is shown in Fig. 1B.

Protein expression and purification of Lol p1 and its variants. Fig. 1A shows SDS/PAGE analysis of expression experiments with recombinant Lol p1 and the three mutants. Inspection of the gel indicated that the recombinant proteins constituted 20–30% of total bacterial proteins. Recombinant Lol p1 remained in the insoluble fraction of the cell homogenate under all growth conditions tested, while the three mutants accumulated mainly in the soluble intracellular fraction (data not shown). Purification of the three mutant proteins was performed by heating the soluble homogenates at 70°C for 10 min, followed by centrifugation to precipitate the bulk of denatured host proteins. To recover recombinant Lol p1, the insoluble cell fraction was dissolved in 25 mM Tris/HCl pH 7.4, 6 M guanidine/HCl and dialyzed against NaCl/P_i. SDS/PAGE analysis of the purified recombinant is shown in Fig. 2A. The higher electrophoretic mobility of recombinant Lol p1 compared with that of natural allergen (Fig. 2A) is consistent with its lack of glycosylation. The yield of recombinant proteins ranged from

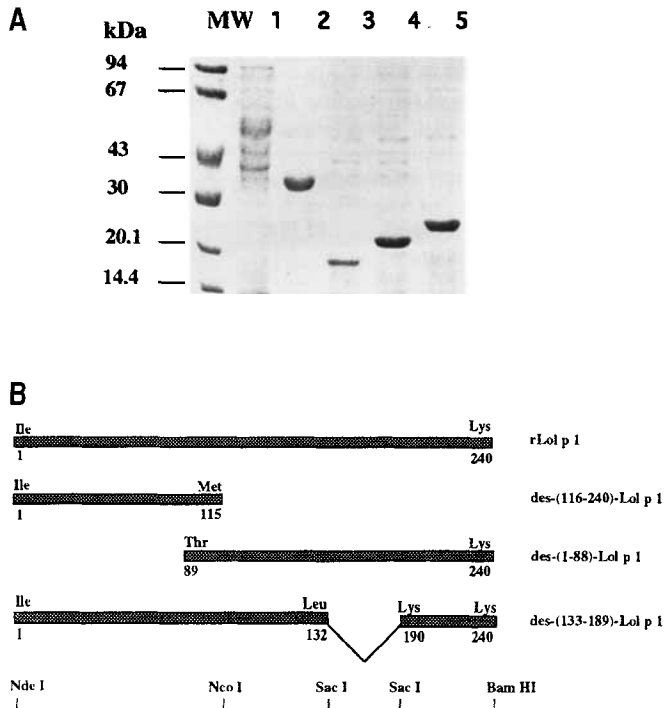


Fig. 1. Expression of recombinant Lol p 1 and its deleted forms. (A) Coomassie-brilliant-blue-stained denaturing SDS/polyacrylamide gel (12.5%) of the total proteins from different recombinant *E. coli* strains. Lane MW, molecular-mass markers; lane 1, negative control [non-transformed BL21(DE3) strain]; lanes 2–5, BL21(DE3) strains transformed with pETLol p 1 (lane 2), pETdes-(116–240)-Lol p 1 (lane 3), pETdes-(1–88)-Lol p 1 (lane 4), and pETdes-(133–189)-Lol p 1 (lane 5). (B) Schematic representation of the recombinant polypeptides and the restriction sites used for the preparation of the mutants. rLol p 1, recombinant Lol p 1.

12 mg/l to 100 mg/l cell culture, with a purity of 80–100%, as estimated from SDS/PAGE (Table 1).

The results of electrospray-mass-spectrometry analysis of purified recombinant Lol p 1 are shown in Fig. 3. The mass of the major component (26238 Da) differs by 43 Da from that calculated from the Lol p 1 sequence with the N-terminal methionine (26195 Da), perhaps as a result of amino acid chemical modification during processing. The mass of the minor component (26113 Da) is 125 Da smaller than the major one, consistent with the removal of the terminal N-terminal methionine.

Immunological characterization of the allergens. Fig. 2B shows a western blot of the allergens, stained with a polyclonal rabbit antiserum raised against recombinant Lol p 1. The specificity of the antiserum is confirmed by the recognition of a single band in the pollen protein extract corresponding to natural Lol p 1. The staining of recombinant Lol p 1 and the three mutants were of comparable intensity. Fig. 2C shows a blot with IgE of a pool of sera from allergic subjects. Several bands corresponding to different allergens were evident in the pollen extract, as expected. Mutants des-(133–189)-Lol p 1 and des-(116–240)-Lol p 1 were stained only very weakly, while des-(1–88)-Lol p 1 as not stained at all, even after extended incubation or exposure time of the filter (data not shown). Thus, the deletion of the first 88 N-terminal residues resulted in the complete loss of IgE recognition on western blots.

To study the immunological reactivity of recombinant Lol p 1 in an ELISA assay, the recombinant protein was absorbed onto microtiter-plate wells, then serial dilutions of sera

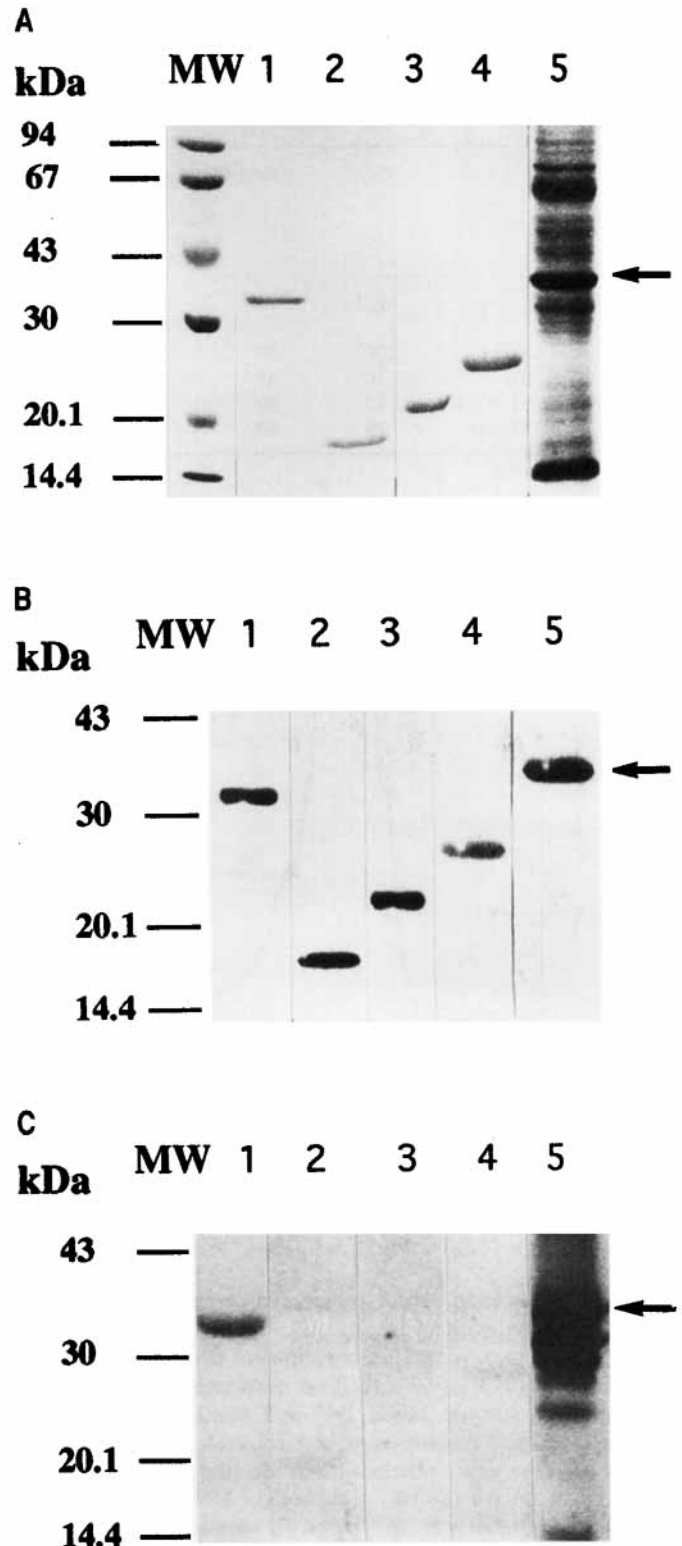


Fig. 2. Analysis of Lol p 1 and mutants. Denaturing SDS/PAGE in 12.5% polyacrylamide gels of the purified recombinant allergens. Lane MW, molecular-mass markers; lane 1, recombinant Lol p 1; lane 2, des-(116–240)-Lol p 1; lane 3, des-(1–88)-Lol p 1; lane 4, des-(133–189)-Lol p 1; lane 5, *L. perenne* pollen extract. (A) Stained with Coomassie brilliant blue. (B) Western blot with rabbit IgG. The samples were transferred to a nitrocellulose filter and incubated with a rabbit polyclonal antiserum raised against recombinant Lol p 1. (C) Western blot with human IgE. The filter was incubated with a pool of sera from subjects sensitized to *L. perenne* pollen. Antibody binding was visualized using HRP-conjugated secondary antibodies. The arrows point to the natural Lol p 1.

Table 1. Biochemical and immunological parameters of recombinant Lol p 1 and mutant allergens. The proteins were characterized for yield (1 l of cell culture is equivalent to about 4 g cell paste), recovery and purity (by densitometry of Coomassie-stained SDS/polyacrylamide gel), molecular mass (by electrospray mass spectrometry), and the capacity to trigger histamine release from sensitized leucocytes (data from Fig. 7, 0.1 µg/ml allergen). The reactivities with IgG and IgE were evaluated by western blotting and with specific ELISA. Peptides b1–b4 showed reactivity in ELISA with IgE and peptides a1, c3, c4, d2, d3 and f1 showed reactivity with IgG. n.d., not determined.

	Yield	Recovery	Purity	Mass	Histamine release	IgE reactivity by		IgG reactivity by	
						western blotting	ELISA	western blotting	ELISA
	mg/l	%		Da	%		%		%
Recombinant Lol p 1	100	50	100	26 238	70	yes	100	yes	100
Des-(1–88)-Lol p 1	20	45	85	n.d.	50	no	27.0	yes	61.5
Des-(116–240)-Lol p 1	12	40	80	n.d.	0	weak	4.5	yes	33
Des-(133–189)-Lol p 1	18	40	90	n.d.	0	weak	6.3	yes	83.3

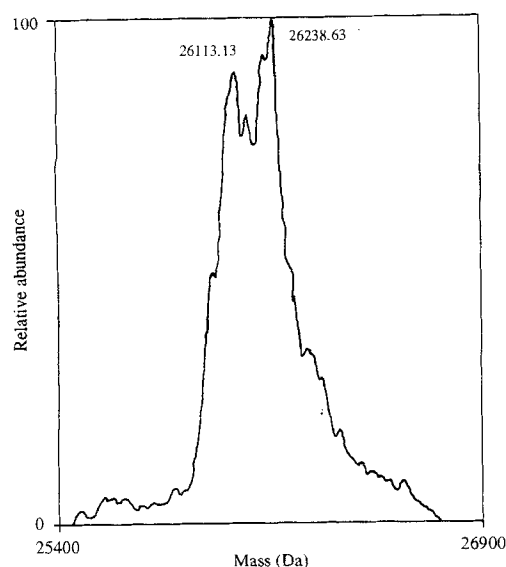


Fig. 3. Mass-spectrometry analysis of the recombinant Lol p 1 polypeptide. Electrospray-mass-spectrometry analysis of purified recombinant Lol p 1. Mass values are reported as average values. The calculated theoretical mass of Lol p 1 with its N-terminal methionine retained is 26 195 Da.

from allergic subjects were overlaid, and the bound IgE antibodies were detected with an IgE-specific secondary antiserum. The response was specific and dose-dependent (Fig. 4A). Similarly, the three mutants were absorbed on microtiter plates and overlaid with the specific rabbit IgG and human IgE antibodies (Fig. 5). The three mutants were recognized by rabbit IgG more weakly than the entire allergen molecule (Fig. 5A) in the order recombinant Lol p 1 (100%) > des-(133–189)-Lol p 1 (83.3%) > des-(1–88)-Lol p 1 (61.5%) > des-(116–240)-Lol p 1 (33.0%). In contrast, only mutant des-(1–88)-Lol p 1 was strongly recognized by IgE, giving a signal with an intensity 27% that of recombinant Lol p 1, and the other two mutants gave signals just above background, with no evident recognition by IgE (Fig. 5B). The data are summarized in Table 1.

Serological characterization using recombinant Lol p 1 ImmunoCAP assays. To examine the prevalence of IgE reactivity to recombinant Lol p 1 in a large sample of allergic subjects and to determine the IgE-binding capacity of the recombinant allergen, recombinant Lol p 1 ImmunoCAP assays were prepared for use in the automated Pharmacia CAP System. A set of 150 sera

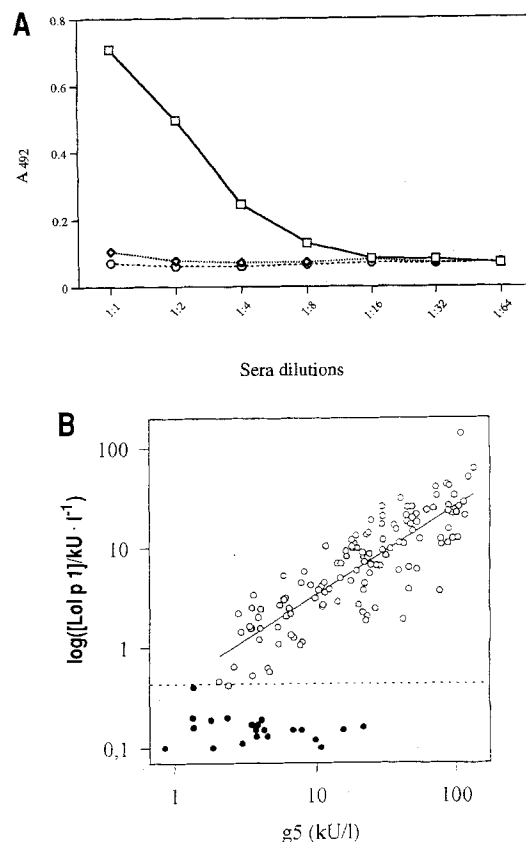


Fig. 4. Determination of specific IgE antibodies for recombinant Lol p 1 in sera. (A) Microtiter-plate wells were coated with recombinant Lol p 1 (10 µg/ml), then incubated with serum samples. Bound IgE was quantitated by incubation with HRP-labelled secondary antibody followed by an enzymatic assay. The response of serial dilutions of the serum from a healthy control (radioallergosorbent test class 0; ○) and from a subject allergic to *L. perenne* (class 6) before (□) and after (◇) incubation with 100 µg/ml recombinant Lol p 1 (2 h at 37°C) are shown. Values are means of duplicate determinations. (B) 150 sera that were radioallergosorbent-positive to *L. perenne* pollen were analysed with g5 ImmunoCAP (*L. perenne* pollen) and recombinant Lol p 1 ImmunoCAP in Pharmacia CAP system. The cut-off value for positivity (0.35 kU/l) is indicated by the dotted line.

with positive radioimmunosorbent tests to grass pollen extract were analysed using the recombinant Lol p 1 and g5 (*L. perenne* pollen extract). In 130 of the sera (87%) IgE recognizing the recombinant allergen was found, which is consistent with previ-

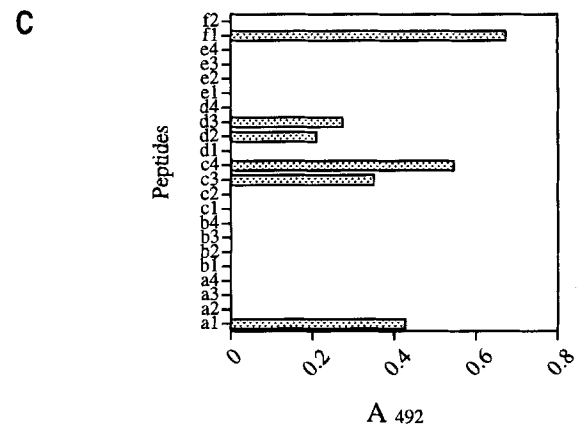
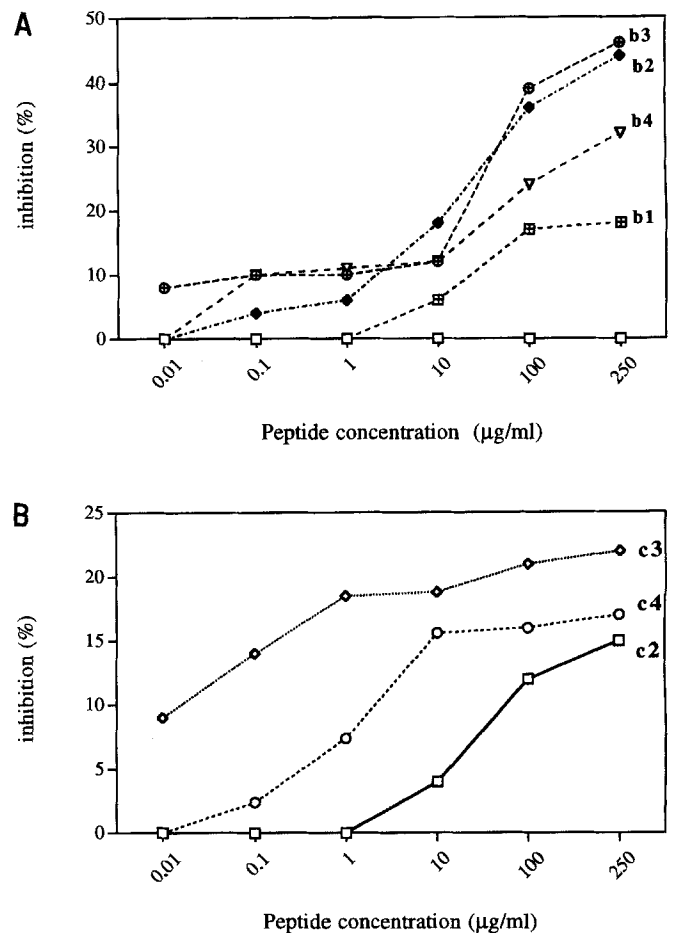
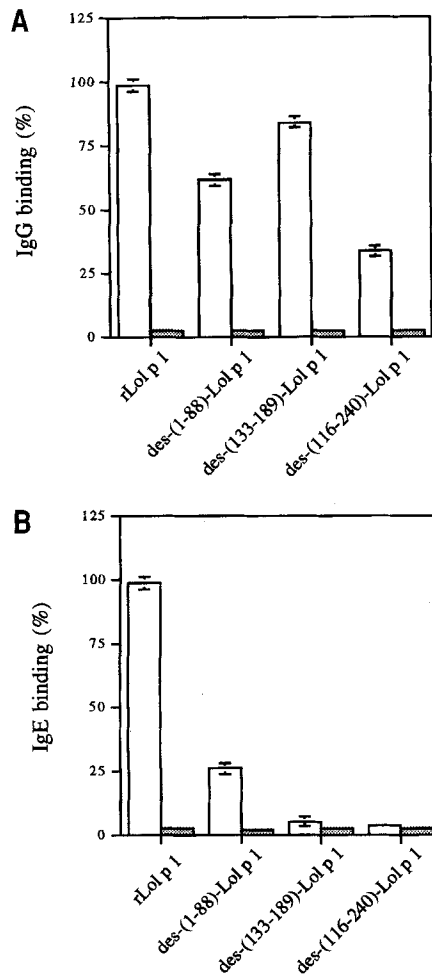


Fig. 6. Antigenic mapping using synthetic peptides. 22 peptides of 17–18 residues covering the whole sequence of recombinant Lol p 1 were synthesized and numbered from the N to C terminus (a–f). They were analysed for antibody-binding capacity in competition (A and B) or in direct (C) assays. (A) Serial concentrations of the peptides were added to sera from allergic subjects (diluted 1:2), the sample added to wells coated with recombinant Lol p 1 and the residual IgE-binding determined with HRP-labelled rabbit anti-(human IgE) IgG. Only the results of the inhibitory peptides are shown, and the results are expressed as percentages of the inhibition relative to that by 2.5 mg/ml recombinant Lol p 1. (B) Experiments analogous to those of panel A, except that the peptides were added to rabbit antiserum for Lol p 1 diluted 1:10000, and residual antibody binding quantified with HRP-labelled goat anti-(rabbit IgG) serum. (C) Microtiter plates were coated with different concentrations of the peptides, incubated with anti-(recombinant Lol p 1) IgG of rabbit antiserum, and the binding was quantified as above. The data show the ELISA response obtained with 1.0 μg/well peptide absorbed onto the solid phase and rabbit antiserum diluted 1:500.

Fig. 5. Immunoreactivity of the mutant allergens. Microtiter-plate wells were coated with 10 μg/ml of recombinant Lol p 1 (rLol p 1), des-(116–240) Lol p 1, des-(1–88)-Lol p 1 or des-(133–189)-Lol p 1, incubated with the sera, and the bound antibodies were revealed with HRP-labelled specific secondary antibodies and development of enzymatic activity. Data are expressed as percentage of the values obtained with recombinant Lol p 1. (A) Incubation with rabbit specific antiserum raised against recombinant Lol p 1 (open bars) and control pre-immune serum (hatched bars), diluted 1:500. (B) Incubation with undiluted human sera from pools of allergic (open bars) and control non-allergic subjects (hatched bars). Values are means of four experiments. Standard deviations are shown as error bars.

ous notions that Lol p 1 is a major allergen to which the vast majority of grass-pollen-sensitized subjects show IgE reactivity. The quantitative IgE values obtained with the recombinant Lol p 1 and g5 tests correlated ($R = 0.67$), and plotting them in a logarithmic form (Fig. 4B) revealed that the sera formed two distinct groups. The larger of these was characterized by high-level IgE to g5 (average 34.0 kU/l) and comprised those sera that were positive in the recombinant Lol p 1 test (average 11.4 kU/l). In this group, Lol p 1 alone appears to be (on average) the target for over 33% of all IgE antibodies for *L. perenne* pollen, provided that g5 ImmunoCAP captures and measures all IgE directed to allergens present in the extract. The second group comprised 20 sera (13%), which lacked IgE to recombinant Lol p 1 and had an average g5 ImmunoCAP value of only 5.6 kU/l. Thus, the presence of IgE antibodies for recombinant Lol p 1 appears to be strongly correlated with high radio-immunosorbent-test levels to total extract of *L. perenne* pollen.

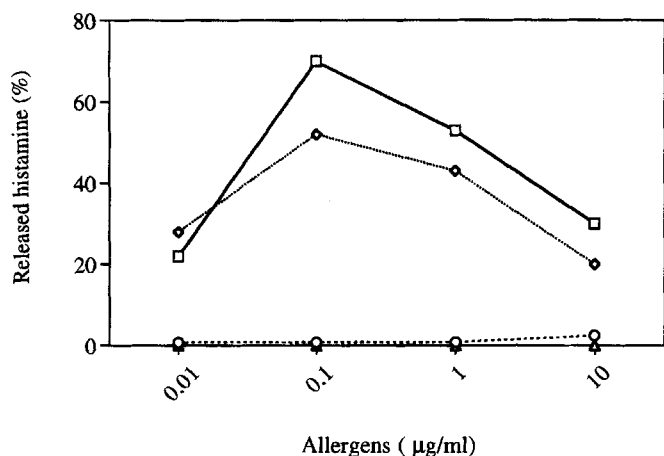


Fig. 7. Histamine-release assay. The blood collected from a class-6 patient was challenged with increasing concentrations of recombinant Lol p 1 (□), des-(116–240)-Lol p 1 (○), des-(1–88)-Lol p 1 (◇) and des-(133–189)-Lol p 1 (△). The histamine released from basophils was determined by immunoassay and expressed as a percentage of the ratio between the released and the total cellular histamine. Data are means of duplicate experiments.

Peptide mapping. To define more precisely the parts of the Lol p 1 molecule contributing to epitopes for human IgE and rabbit IgG, we synthesized overlapping peptides of 17–18 residues covering the whole sequence of Lol p 1. The peptides were used in ELISA to test their capacity to compete with recombinant Lol p 1 for antibody binding. The inhibition plots (Fig. 6A) show that peptides b1, b2, b3 and b4, which cover the region between residues 42 and 88 of the protein sequence, could significantly displace binding of human IgE binding to the absorbed recombinant Lol p 1. The most active peptides (b2 and b3, residues 53–80) produced around 35–40% inhibition at a concentration above 100 µg/ml. None of the other peptides caused detectable specific inhibitions, even at higher concentrations (data not shown).

Binding of rabbit IgG to recombinant Lol p 1 was significantly inhibited only by peptides c2, c3 and c4 (Fig. 6B), with peptides c3 and c4 (residues 95–128) displacing more than 15–18% of the signal at concentrations above 10 µg/ml. In a separate set of experiments, the peptides were absorbed on the wells and the direct binding of rabbit IgG analysed. The results obtained from this assay confirmed the binding capacity of peptides c3 and c4 and revealed activity of peptides a1 (residues 1–17), d2, d3 (residues 134–161) and f1 (residues 213–230) (Fig. 6C). When the same type of ELISA was performed with human serum, none of the peptides was found to bind IgE (data not shown).

Histamine release. To examine the allergenic activity of recombinant Lol p 1 and the deletion mutant derivatives, basophils from an allergic subject (E. T.) were challenged *in vitro* with various concentrations of purified proteins and the amount of released histamine measured (Fig. 7). Recombinant Lol p 1 induced a maximum release of 70% total histamine at 0.1 µg/ml, and the N-terminally deleted des-(1–88)-Lol p 1 produced a maximum release of 50% total histamine at 0.1 µg/ml, while the other two mutants did not trigger any detectable response over a wide concentration range (0.01–10 µg/ml).

DISCUSSION

Biochemical and immunological properties of recombinant Lol p 1 and mutants. We describe a procedure to produce re-

combinant non-fused Lol p 1 with yields of about 100 mg pure protein/l fermentation broth (Table 1). Electrospray-mass-spectrometry analysis confirmed that the expressed protein is the correct one, partially deleted *in vivo* of the N-terminal residue (Fig. 3), and with a probable chemical modification occurring during *in vitro* manipulations. The recombinant protein binds to serum IgE of sensitized subjects (Fig. 2), induces histamine release from basophils of sensitized subjects (Fig. 7), and elicited rabbit antisera that recognize specifically Lol p 1 in pollen extracts (Fig. 2B). We concluded that recombinant Lol p 1 is an allergen, similar to natural Lol p 1.

The three mutants bound rabbit IgG, and the reactivities in ELISA of 33%, 62% and 83% that of recombinant Lol p 1 were related to the sizes of the deletions: 51%, 37% and 23% of the sequence for the three mutants (Fig. 5A). Analysis of reactivity with IgE was more complex. Mutants des-(133–189)-Lol p 1 and des-(116–240)-Lol p 1 were recognized weakly in western blots and even more weakly in ELISA, and consistently, they did not induce histamine release from sensitized cells. Thus, these two proteins do not behave as true allergens in these analyses. The other mutant, des-(1–88)-Lol p 1, gave strong signals in ELISA and elicited significant histamine release from sensitized cells (Fig. 7), although it was not recognized by IgE in western blotting. We concluded that the mutant has notable allergenic properties.

Epitope mapping. The finding that rabbit IgG binding decreased with the extent of the mutant deletions indicated the presence of many binding sites distributed on the molecule. To locate these more precisely, we used synthetic peptides in competition (Fig. 6B) and in direct ELISA (Fig. 6C) experiments. A major epitope in the region 134–161 was identified by both approaches, while epitopes at residues 1–17 and 213–230 were indicated only by the direct binding assay. These data support and extend previous reports demonstrating that the C-terminal sequence, residues 216–240, of Lol p 1 was recognized by human IgE and IgG and by rabbit IgG elicited by immunotherapy with grass pollen extracts and by immunization with purified Lol p 1, respectively (van Ree et al., 1994), and that rabbit IgG directed to residues 216–240 inhibited Lol p 1-mediated histamine release from human basophils to about 50% (Ansari et al., 1989).

The low titer and high heterogeneity of human IgE (which may differ among subjects) make epitope mapping rather complex. Using a pool of sera from allergic subjects in competition experiments with synthetic peptides (Fig. 6A) we found a major sequential IgE-binding determinant on the region 42–88 (peptides b1–b4). This extends previous finding by van Ree et al. (1994; 1995) of a C-terminal structure of Lol p 1 that bound IgE from serum of one selected subject.

Our results are consistent with the finding that des-(1–88)-Lol p 1 is not recognized by human IgE antibodies in western blotting, while the other two mutants, which contain residues 1–88, are recognized, although weakly (Fig. 2C). Since both approaches, peptide mapping and blotting of denatured proteins, are aimed to recognize sequential epitopes and gave consistent results, we concluded that the sequence 42–88 carries an important sequential epitope. However, as indicated above, the two mutants with the 42–88 sequence did not behave as allergens under native, non-denaturing conditions (i.e. ELISA and histamine release), suggesting that the epitope is not accessible to IgE in the 'folded' molecules. It is conceivable that due to the extensive deletions the two mutants assumed unnatural conformations that masked the epitope. More significant is the finding that the mutant missing this sequence is the only one acting as a true allergen, which suggests that it exposes conformational

epitopes undetectable by blotting and peptide mapping. The results lead to the suggestion that in Lol p 1 conformational IgE epitopes are more important than the sequential ones.

This hypothesis is supported by previous evidence that a sequence of Phl p 1 highly similar to residues 101–115 of Lol p 1 bound IgE attached to the C-terminus of β -galactosidase but not when prepared in a synthetic form (Ball et al., 1994). Apparently, the conformation or presentation context played a major role in IgE binding.

Recombinant Lol p 1 ImmunoCAP. While western blot analysis of sera using allergen extracts is limited by the resolving power of the electrophoretic system and, at best gives a semi-quantitative result, access to pure, recombinant allergens offers the possibility to establish fully quantitative immunoassays for serum characterization. To explore this option, we prepared a series of recombinant Lol p 1 ImmunoCAP for use in the Pharmacia CAP System. Using these tests, together with the g5 ImmunoCAP (carrying *L. perenne* pollen extract) we examined sera from 150 grass-pollen-sensitized subjects. In 130 of these sera (87%), IgE reactivity to recombinant Lol p 1 was found, at an average level of 37% of the IgE value obtained with the g5 ImmunoCAP. Thus, we demonstrate that this bacterially produced grass pollen allergen exhibits quantitatively potent IgE binding and is recognized by serum IgE from the vast majority of sensitized subjects. The results confirm the importance of Lol p 1 as a major allergen (Kahn and Marsh, 1986; Bond et al., 1993).

A peculiar outcome of the serological study was the observation of a strong correlation between the presence of IgE antibodies recognizing recombinant Lol p 1 and high-level IgE to total extract of *L. perenne* pollen. In sera that contained IgE to recombinant Lol p 1, the average g5 value was 34.0 kU/l, while sera that were negative with the recombinant Lol p 1 had an average g5 level of only 5.6 kU/l. This observation would seem to suggest that either IgE reactivity to Lol p 1 is strongly associated with reactivity to other grass pollen allergens (which would account for the non-recombinant Lol p 1 portion of the IgE values obtained with g5 ImmunoCAP), or that the recombinant Lol p 1 ImmunoCAP has the capacity to detect only a certain fraction of the IgE antibodies that recognize natural Lol p 1 allergen present in the g5 ImmunoCAP. Although we cannot distinguish between these two possibilities on the basis of our data, because allergens other than Lol p 1, such as Lol p 2 and Lol p 5, also contribute substantially to IgE-binding activity in *L. perenne* pollen extract (Freidhoff et al., 1986; Bond et al., 1993), it seems unlikely that the high g5 ImmunoCAP value of the sera reactive to recombinant Lol p 1 could be entirely accounted for by natural Lol p 1 present in the g5 test, which the recombinant Lol p 1 test fails to fully reproduce. Instead, it is reasonable to assume that at least part of the reason for the observation is a genuine association between reactivity to Lol p 1 and reactivity to other allergens. While such a phenomenon may appear puzzling, a rational explanation could be that the comparatively low g5 ImmunoCAP value found in those sera that were negative with the recombinant Lol p 1 test does not represent a true sensitization to *L. perenne* pollen in these serum donors, but a moderate level of cross-reactivity to different allergens, not involving Lol p 1. If this is true, then the prevalence of IgE reactivity to recombinant Lol p 1 among subjects that are genuinely sensitized to *L. perenne* pollen may be even greater than the 87% found in our screen of 150 sera.

In conclusion, recombinant Lol p 1 can be produced in large amounts, it has allergenic properties and is useful for the evaluation of specific IgE in allergic patients. Its IgE epitopes appear to be mainly conformational and can be inactivated by extensive

deletions, which modify the protein without eliminating with IgG. This indicates that IgE-binding and IgG-binding sites are not overlapping, as has been shown for Lol p 2 using an analogous approach (de Lalla et al., 1996). This finding indicates the possibility to produce molecules that elicit a protective IgG response without being allergenic. Des-(116–240)-Lol p 1 may be particularly interesting since it lacks a major T-cell epitope (located in the sequence 190–210) described in various reports (Perez et al., 1990; Bungy et al., 1993, 1994; Baskar et al., 1992; Spiegelberg et al., 1994). The present study encourages the production of allergen molecules with modified allergenic properties, which can be further manipulated to create safer and more effective reagents for specific diagnosis (Pauli et al., 1996) and immunotherapy (Dickler and Collier, 1996).

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