

Original Article

Effect of cysteine mutagenesis on human IgE reactivity of recombinant forms of the major rye grass pollen allergen Lol p 1

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ABSTRACT

Background: Hypersensitivity to the group 1 grass pollen allergens is a significant causative factor in the onset of symptoms for hay fever sufferers. To better understand the IgE reactivity of the group 1 allergen from rye grass pollen, we sought to disrupt potential conformational IgE epitopes on recombinant (r) Lol p 1 by the specific replacement of the seven cysteine residues in the protein sequence.

Methods: Site-directed mutagenesis on the Lol p 1 coding sequence was used to replace all seven cysteine residues with serine residues. rLol p 1 and the seven cysteine variants generated by this method were tested for comparative human IgE reactivity via western blot immunoscreening and densitometry.

Results: Alteration of the cysteine residues at amino acid positions 72, 77, 83 and 139 of rLol p 1 was found to reduce the human IgE binding potential of the molecule. However, the most consistent reduction in human IgE reactivity was demonstrated by replacement of C⁷⁷; human IgE antibodies showed an average 62.7% reduction in reactivity to this molecule.

Conclusions: The present investigation has shown that at least one of the cysteine residues within the Lol p 1 protein contributes to the IgE binding properties of this allergen.

Key words: allergy, grass pollen, Lol p 1, mutagenesis, rye grass.

INTRODUCTION

The grass group 1 pollen allergens, glycosylated proteins of approximately 28–35 kDa,¹ are major allergens with up to 95% of grass pollen allergic individuals showing immunoreactivity.² Because homologous proteins have been identified in all grass species tested, the group 1 grass pollen allergens are an important group of immunoreactive proteins.^{3–6} Cloning and sequencing of the group 1 pollen allergens from eight grass species has revealed significant levels of amino acid conservation, including the presence of seven strictly conserved cysteine residues within these proteins.

Although functionally and immunologically distinct, the group 1 grass pollen allergens show conservation of six of their seven conserved cysteine residues and 25% overall amino acid homology to expansins, a group of proteins that induce plant cell wall extension (creep).⁷ On the basis of the extent of amino acid conservation between the two groups of proteins, the group 1 grass pollen allergens are predicted to share a similar secondary structure to the expansins.⁸ Furthermore, the degree of cysteine conservation also suggests a common pattern of disulfide bond formation and protein folding between the two groups of proteins. An expansin-like activity has also been demonstrated for the purified group 1 allergen from maize pollen.⁸ Amino acid sequence homology has been shown between the group 1 allergens and three functionally important sequence motifs of the C1 (papain-like) family of cysteine proteinases.⁹ Recently, Grobe *et al.*¹⁰ have shown that the group 1 pollen allergen from Timothy grass demonstrates similar biochemical

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and functional properties to cathepsin B, a member of the papain (C1) family of cysteine proteinases.

Although the presence of disulfide bonds within group 1 grass pollen allergen molecules has not been demonstrated specifically, homologies with functional proteins, such as the expansins and C1 cysteine proteases, suggest the conservation of certain structural properties, possibly stabilized by the presence of intramolecular disulfide bonds. In certain allergen molecules, intramolecular disulfide bonds participate in the presentation of conformational IgE epitopes on the molecular surface.¹¹ Indeed, for the group 2 allergens from house dust mites, disulfide bonds have been shown to assist in the formation of conformational IgE epitopes on the molecular surface.^{12,13} For Lol p 1, the group 1 pollen allergen from rye grass, a number of linear IgE-binding epitopes have been mapped using either synthetic peptides corresponding to linear segments of the allergen or a pool of monoclonal antibodies to block human IgE binding.^{14–17} Although certain linear regions of rLol p 1 have been shown to be involved in human IgE antibody interaction, the presence of potential conformational epitopes on the molecular surface of this allergen, as mediated by intramolecular disulfide bridges between cysteine residues, is unknown.

Immunotherapeutic treatment of inhalant allergies traditionally involves the subcutaneous administration of increasing doses of a crude allergen extract consisting of impure and poorly characterized, allergenic and non-allergenic proteins.¹⁸ Due to the high IgE-binding properties of native allergens, administration of such crude protein preparations to sensitive individuals carries the risk of recipients developing a potentially life-threatening anaphylactic response.¹⁹ With the aim of developing allergen-specific immunotherapeutic reagents, recent studies (see Discussion) have shown the production of highly purified recombinant forms of native allergens with reduced IgE-binding capacity and anaphylactogenic potential. The targeted alteration of recombinant allergens from house dust mite feces (Der f 2, Der p 2) have recently reported the successful use of site-directed mutagenesis for the replacement of cysteine residues to reduce or abolish the IgE reactivity of these proteins.^{20,21} In the present investigation, we sought to determine the importance of the cysteine residues of rLol p 1 to the overall IgE binding capacity of this recombinant allergen and, in doing so, generate potentially hypoallergenic forms of this major rye grass pollen allergen.

METHODS

Cloning into the expression vector

The pMAL-c2 expression vector (Qiagen, Hilden, Germany) was used for the generation of all rLol p 1 and rLol p 1 variant proteins. The cDNA encoding *Lolp1*, previously cloned from a cDNA expression library,²² was amplified using specific primers designed to introduce restriction sites at both ends of the coding region (5' primer: GCAGCGCGCATGATATCGCGAAGG; 3' primer: CACTCCACTGAATTCTCACTGGCCGAG). The *EcoRV* restriction site introduced into the 5' end of the coding region allowed the in-frame ligation into the *XmnI* site in the pMAL-c2 multiple cloning site. The position of the *EcoRV* site was designed specifically to permit factor Xa cleavage of the expressed Maltose-binding protein (MBP)–allergen fusion complex, allowing the allergen to be released from the MBP without the addition of extraneous amino acids. DNA sequencing of the junction between the *malE* and *Lolp1* coding regions confirmed the correct 5' sequence for *Lolp1*. All variant constructs were generated by site-directed mutagenesis of the pMAL–*Lolp1* vector.

Site-directed mutagenesis of *Lolp1*

The *Lolp1* gene in pMAL-c2 was altered by site-directed mutagenesis at each of the seven cysteine residues within the Lol p 1 protein (see Fig. 1). In all cases, primers were designed to alter the coding sequence from TGC to TCC, with the new codon encoding serine.

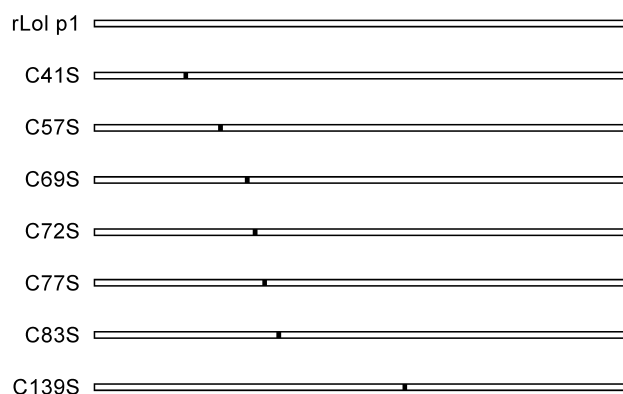


Fig. 1 Linear representation of rLol p 1 and the seven cysteine variants (C41S, C57S, C69S, C72S, C77S, C83S and C139S) showing the approximate location of the cysteine residues that were altered within each molecule.

The cysteine variants of rLol p 1 are identified according to the residue altered and, thus, the variant with the cysteine residue at amino acid residue position 41 altered to serine was identified as C41S, the variant altered at amino acid residue position 57 was identified as C57S etc. (see Fig. 1). The forward primers used for the site-directed mutagenesis of the *Lolp1* sequence are listed hereafter: to generate C41S variant, CGGCGGCGCGTCCGGGTACAAG; C57S GCATGACCGGCTCCGGCAACAC; C69S CGGCCGTGGCTCCGGCTCCTGC; C72S CTGCGGCTCCTCCTTGAGATC; C77S GAGATCAAGTCCACCAAGCCCG; C83S GCGCGAGTCTCTCCGGCGAG; and C139S GGGTCAAGTCCAAGTACCCGG. Reverse primers were the reverse complement of the forward primers listed. Site-directed mutagenesis was performed using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA, USA) in a volume of 50 μ L containing 0.05 mmol/L each dNTP, 125 ng each of the forward and reverse primers, 2.5 units *Pfu* polymerase (Promega, Madison, WI, USA) and the appropriate dilution of the supplied 10x buffer. All reactions consisted of an initial denaturation step of 95°C for 30 s, 16 cycles of 95°C for 30 s, 55°C for 30 s and 68°C for 2 min per kb plasmid and then cooling to 4°C. All reactions were performed in a GeneAmp 2400 PCR system (Perkin Elmer, Norwalk, CT, USA). After completion of the reaction, 2 μ L (20 units) restriction enzyme *DpnI* was added and the preparation incubated at 37°C for 3 h before transformation of an aliquot into freshly prepared competent XL1B cells prepared according to the method of Cohen *et al.*²³

Expression and purification of proteins fused to MBP

A 500 mL aliquot of LB broth containing 0.2% (w/v) glucose and 100 μ g/mL ampicillin (Sigma Aldrich, St Louis, MO, USA) was seeded with 10 mL overnight culture of XL1B cells (Stratagene) housing the appropriate plasmid and grown at 37°C for 4 h. Isopropyl- β -D-thiogalactoside was then added to a final concentration of 0.3 μ mol/L to induce expression of the fusion protein. Following incubation at 37°C for a further 2 h, bacterial cells were harvested by centrifugation at 3840 g for 10 min. Cells from one 500 mL culture were resuspended in 20 mL column buffer (20 mmol/L Tris buffer (pH 7.4), 200 mmol/L sodium chloride, 1 mmol/L EDTA) and stored at -20°C overnight. Resuspended cells were

thawed slowly in cold water before sonication six times for 15 s each (15 s on, 15 s off) in an ice bath at 20 μ m peak-to-peak using an Mk2-6-76 Sonicator (MSE, Loughborough, UK). The sonicated preparation was centrifuged at 9000 g for 20 min at 4°C. The supernatant was harvested and added to 3 mL prewashed amylose resin (New England Biolaboratories, Beverly, MA, USA). The mixture was rocked gently on ice for 1 h before centrifugation for 10 s at 700 g to pellet the resin. After resuspension in 15 mL column buffer, the resin and protein were rocked gently on ice for 10 min. Centrifugation and washing were performed twice before the resin was poured into a disposable 1 mL column (Qiagen). The resin column was allowed to form by gravity before washing with a further six column volumes of column buffer. Fusion proteins were eluted from the column in two volumes of column buffer supplemented with 10 mmol/L maltose. Fusion proteins were visualized by Coomassie staining of 12.5% (w/v) sodium dodecyl sulfate (SDS)-polyacrylamide gels.²⁴ The concentration of proteins present in eluted aliquots was determined according to the method of Bradford²⁵ and confirmed by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie staining against a protein standard of known concentration.

Factor Xa treatment of fusion proteins

Factor Xa used for digestion of MBP from rLol p 1 and the cysteine variants was purchased from New England Biolaboratories and used at a concentration of 10 μ g/mg fusion protein, with digestion allowed to proceed at 4°C with gentle rotating for 5 h. Electrophoresis and Coomassie staining of a 12.5% (w/v) SDS-polyacrylamide gel was used to verify digestion. Digested samples were stored at -20°C before use.

Electrophoresis and western blotting of rLol p 1 variants

Approximately 2 μ g total protein of each of rLol p 1 and the seven cysteine variants, after factor Xa treatment, were separated by SDS-PAGE (15% w/v)²⁴ on a mini-Protean II protein gel system (Bio-Rad, Hercules, CA, USA) in a buffer of 200 mmol/L glycine, 0.1% (w/v) SDS and 25 mmol/L Tris buffer (pH 6.8). In a dye composed of 0.125 mol/L Tris buffer (pH 6.8), 20% (v/v) glycerol and 50 mmol/L bromophenol blue, proteins were separated under non-reducing conditions without prior heat

denaturing treatment.²⁴ Prior to blotting, a duplicate gel was tested for the presence of proteins by SDS-PAGE and Coomassie staining. Duplicate gels were blotted to pretreated polyvinyl difluoride (PVDF) membrane (Osmonics, Westborough, PA, USA) using a mini-Protean II electroblotting system in a buffer of 200 mmol/L glycine, 25 mmol/L Tris buffer and 20% (v/v) methanol at 100 V for 70 min. After drying, blots were blocked in 10% (w/v) milk powder in phosphate-buffered saline (PBS; 0.15 mol/L sodium chloride, 3.6 mmol/L sodium dihydrogen orthophosphate, 6.7 mmol/L di-sodium hydrogen orthophosphate) for 1 h at room temperature with shaking. After washing twice in PBST (PBS with 0.05% (v/v) Tween 20) and twice in PBS for 10 min each, blots were incubated in sera from grass pollen-allergic patients diluted 1 : 5 in PBS containing 1% (w/v) bovine serum albumin (BSA) and 0.02% (w/v) sodium azide. After overnight shaking at room temperature, blots were washed as above and incubated with an alkaline phosphatase-conjugated goat, antihuman IgE antibody (diluted 1 : 1000 in PBS containing 1% (w/v) BSA and 0.02% (w/v) sodium azide; Sigma). After 2 h shaking at room temperature, blots were again washed as above and incubated in 0.4 mmol/L nitroblue tetrazolium (Boehringer, Mannheim, Germany), 0.4 mmol/L 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (Astral Scientific, Gymp, NSW, Australia) prepared in a solution of 100 mmol/L Tris buffer (pH 9.5), 100 mmol/L sodium chloride and 50 mmol/L magnesium chloride. Color development was halted by incubation in 0.1 mol/L EDTA. For chemiluminescence assay, blots were developed using CDP-Star reagent (Boehringer, Mannheim, Germany) and exposing to Kodak MR film (Integrated Sciences, Melbourne, Victoria, Australia). Image densities were subjected to quantification using Kodak EDAS 120 digital imaging software.

RESULTS

Reduced IgE-binding capacity of rLol p 1 cysteine variants as tested by western blot analysis

Upon western blotting and immunoscreening of rLol p 1 and the cysteine variants with an anti-Lol p 1 monoclonal antibody and a rabbit polyclonal antisera, it could be seen that the protein products at approximately 33 kDa bound both antibodies (Fig. 2). Furthermore,

the intensity of binding of the antibodies was similar for rLol p 1 and the seven cysteine variants, indicating that the same amount of protein product at the size expected was present for each of the proteins. Immunoscreening with sera from grass pollen-allergic patients showed the differing abilities of the rLol p 1 cysteine variants to bind human IgE antibodies (Fig. 2). Although the rLol p 1 cysteine variants C41S, C57S and C69S appeared to bind human IgE antibodies at an equivalent or greater level than rLol p 1, variants C72S, C77S, C83S and C139S showed a reduced capacity to bind human IgE antibodies from the sera of certain patients (Fig. 2). The rLol p 1 cysteine variant C77S showed the lowest level of human IgE antibody binding of the proteins immunoscreened with sera from grass pollen-allergic patients MS, TP and JL (Fig. 2). When rLol p 1 and the seven cysteine variants were immunoscreened with the diluted sera from patient DI, cysteine variant C83S showed the lowest level of human IgE antibody binding. After incubation with the sera from patient RT, human IgE antibody binding to cysteine variants C72S, C77S, C83S and C139S was not visible by the method used.

Western blots were subjected to densitometric analyses to obtain semiquantitative data for the level of human IgE antibody binding to rLol p 1 and cysteine variants. It is interesting to note that, for most patients, variant C57S showed slightly higher level of IgE reactivity compared with rLol p 1. However, cysteine variants C72S, C77S, C83S and C139S showed reduced IgE reactivity in most cases. Of all the variants tested, mutant C77S showed most pronounced reduced IgE-binding capacity compared with rLol p 1 in all five patient sera tested (Table 1). One patient (RT) showed as little as 12.5% human IgE antibody binding to C77S compared with rLol p 1.

DISCUSSION

More than 90% of grass pollen-allergic patients possess IgE antibodies that recognize the group 1 pollen allergens.² Furthermore, because proteins homologous to these allergens have been identified in pollen extracts from all grasses tested, it is clear that this group of inhalant allergens has a major impact on the incidence of grass pollen allergy. The present study investigated the impact that the seven strictly conserved cysteine residues of Lol p 1 have on the human IgE-binding capacity of the recombinant allergen.

Studies predicting the IgE epitopes of Lol p 1 have previously used either synthetic peptides, corresponding to

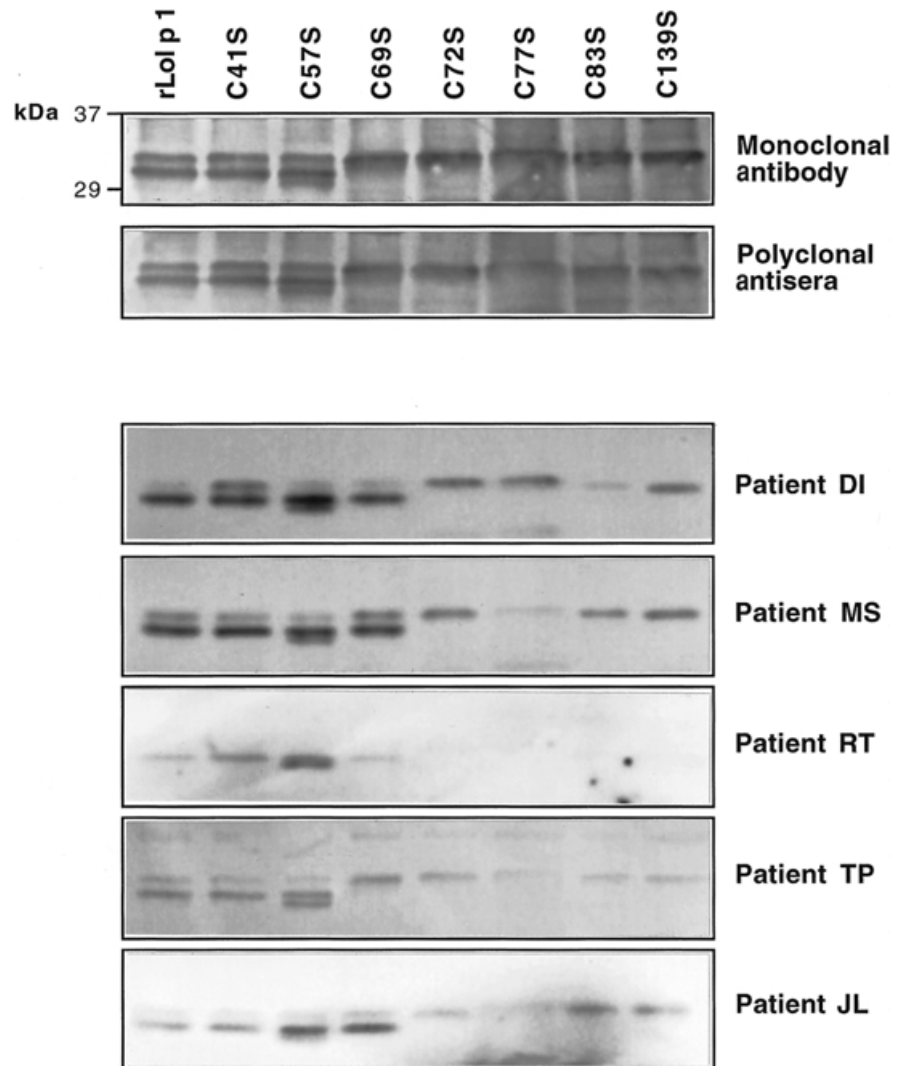


Fig. 2 Western blot immunoscreening of rLol p 1 and the seven cysteine variants (C41S, C57S, C69S, C72S, C77S, C83S and C139S) with an anti-group 1 grass pollen allergen monoclonal antibody and polyclonal antisera, and sera from five grass pollen-allergic patients (DI, MS, RT, TP and JL). Proteins of between 29 and 37 kDa are shown on each blot.

Table 1 The level of human IgE binding to various cysteine mutants expressed as a percentage of binding to rLol p 1

	Cysteine variant						
	C41S	C57S	C69S	C72S	C77S	C83S	C139S
Patient							
DI	102.2	110.4	92.5	80.4	76.8	62.9	76.0
MS	104.0	102.5	65.0	77.0	26.0	70.5	79.0
RT	110.5	130.0	100.0	18.8	12.5	15.5	16.0
TP	100.0	104.0	99.0	91.5	39.0	35.0	35.0
JL	115.0	143.0	143.0	104.5	37.0	105.0	97.0
Mean	106.3	118.0	100.0	74.5	38.3	56.8	59.8

The level of human IgE antibody binding to the C77S variant is significantly reduced from that binding to rLol p 1.

linear segments of this allergen, or a pool of monoclonal antibodies to block human IgE binding to linear peptides.¹⁴⁻¹⁷ The use of linear synthetic peptides and monoclonal antibodies to determine the position of IgE

epitopes on allergen molecules is limited in that the presence of conformational epitopes could be overlooked. Evidence for the existence of at least one conformational IgE epitope for Lol p 1 can be gained from a previous

study using recombinantly expressed Lol p 1 fragments to inhibit human IgE binding to rLol p 1.¹⁷ In this study, two fragments of Lol p 1, corresponding to amino acids 1–168 and 169–240, could inhibit human IgE antibody binding to rLol p 1 by 17 and 51%, respectively.¹⁷ Because these fragments together represent the complete Lol p 1 molecule, and no known linear IgE epitope has been mapped across their point of junction, the remaining 32% of human IgE binding not inhibited by these fragments suggests the presence of at least one conformational IgE epitope. The results of the present study also suggest that one or more of the cysteine residues present within Lol p 1 may contribute to the formation of a conformational IgE-binding epitope on this allergenic protein.

Site-directed mutagenesis of cysteine residues within rDer p 2 and rDer f 2 has been demonstrated to reduce the allergenic reactivity of both recombinant house dust mite allergens.^{20,21} These studies not only demonstrate the importance of disulfide bonds to IgE epitope formation for these proteins, but they also show that disruption of key disulfide bonds can dramatically reduce the allergenic nature of these molecules. Unlike the group 2 house dust mite allergens, the presence of intramolecular disulfide bonds has not been proven for the group 1 grass pollen allergens. However, the presence of seven strictly conserved cysteine residues in the protein backbone, along with sequence and predicted structural homologies to functional proteins, implies that the group 1 grass pollen allergens possibly have a conserved molecular structure, which may be influenced by disulfide bonds. Our results show that replacement of cysteine residues at positions 41, 57 and 69 has no negative effect on IgE reactivity of rLol p 1. In fact, variant C57S showed higher levels of IgE reactivity compared with rLol p 1. It is possible that mutation of C57 may have altered the conformation of the rLol p 1 molecule in such a way that the linear IgE-binding epitopes of the allergen became more accessible to these antibodies, thereby increasing the IgE reactivity of the C57S variant. The results of the present study suggest that residue C77 of rLol p 1, and also perhaps those situated at amino acid residue positions 72, 83, and 139, may contribute to the formation of a structure that is critical for human IgE recognition of rLol p 1. If cysteine at position 77 was involved in disulfide bond formation, then another counterpart derivative should have shown similar reduction in IgE reactivity. Because no counterpart derivative was observed, it is possible that cysteine at position 77 is critical in IgE

epitope formation but that it may not participate in disulfide bond formation, whereas the other six cysteine residues may form disulfide bonds. As discussed above, it is also possible that mutation of selected residues may change the conformation in such a way that linear IgE-binding epitopes of Lol p 1 become more/less accessible to human IgE antibodies. Patient-to-patient variability and the documented presence of linear human IgE epitopes on rLol p 1 may, together, have led to an inconsistent reduction in human IgE binding to 'the counterpart' derivative involved with disulfide bond formation with cysteine residue 77. The results of the present study also suggest that individual cysteine residues are not solely responsible for presentation of the immunodominant IgE epitope of rLol p 1. It is apparent from these results that other factors, perhaps the presence of linear IgE epitopes, may also play a role in human IgE recognition of rLol p 1.

The immunodominant T cell epitope of Lol p 1 has been identified as the peptide spanning amino acid residues 193–206.^{26–28} This peptide, along with a second mapped T cell epitope spanning amino acid residues 215–229, has been retained in all rLol p 1 variants generated in the present study. Furthermore, the immunodominant T cell epitope of Phl p 1, the group 1 pollen allergen from Timothy grass, has been identified as the peptide spanning amino acids 70–84 of this protein.²⁹ Lol p 1 and Phl p 1 share 14 identical amino acids in this peptide, including three of the seven cysteine residues strictly conserved in all group 1 grass pollen allergens. This peptide is altered in cysteine variants C72S, C77S and C83S; each of these molecules contains one amino acid residue that differs from the reported sequence.³⁰ The effect of these cysteine residues on peptide recognition by T cells is undetermined and, therefore, these rLol p 1 cysteine variants would need to be tested experimentally to demonstrate the effect of cysteine mutagenesis on the ability to stimulate T cell clones recognizing this region.

Based on the hypothesis that decreasing IgE antibody binding but maintaining T cell recognition of allergens will decrease the risk of anaphylaxis while maintaining the ability to induce an immune response, hypoallergenic variants of recombinant allergens are now being developed for use in specific immunotherapy of pollen allergic diseases. A number of studies have suggested that specific immunotherapy can be effective when the balance of cytokines released from T cells is altered with a shift from an allergic (Th2) to an immunogenic (Th1) response.^{31,32}

Thus, non-IgE-binding forms of allergens may, in their capacity to be recognized by T cells but not IgE antibodies, induce a shift in the helper T cell response from Th2 to Th1. Site-directed mutagenesis has been used successfully to produce low IgE-reactive recombinant forms of house dust mite (Der p 2, Der f 2) and pollen allergens from rye grass (Lol p 5), birch (Bet v 1, Bet v 4) and oilseed rape (Bra r 1).^{20,21,33–36} These results have shown that replacement of very few key amino acids within an allergen molecule can significantly reduce the human IgE reactivity of these allergenic proteins. The results of the present study may aid the development of a recombinant form of Lol p 1 with reduced human IgE reactivity and, therefore, may contribute to the development of improved immunotherapeutic reagents for the treatment of grass pollen allergy.

Although the presence of cysteine residues on the Lol p 1 protein has been known since the cDNA was cloned and characterized, the specific contribution these residues make to the allergenicity of the protein has not been reported. The outcomes of the present study suggest that at least one of the cysteine residues on the Lol p 1 molecule contributes to the allergenicity of this protein, either by participating in the formation of a conformational epitope or in the uniform presentation of linear epitopes. The group 1 grass pollen allergens are major contributors to grass pollen allergy in many parts of the world. Elucidation of the influence of cysteine residues to allergenicity of rLol p 1 is important for a thorough understanding of the immunological properties and allergenic nature of this allergen. Application of this knowledge, by the generation of low IgE-binding forms of Lol p 1, may aid the future development of improved immunotherapeutic reagents for the treatment of grass pollen allergy in individuals sensitive to the group 1 grass pollen allergens.

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