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Immunological and Biological Properties of Recombinant Lol p 1

Abstract

Background: Current forms of allergy diagnosis and therapies are based on the use of natural allergenic extracts. Despite strong evidence that higher therapeutic efficacy may be achieved with purified allergens, the purification of multiple allergic components from extracts is a fastidious and sometimes an impossible task. However, the use of recombinant allergens may be an alternative to overcome this problem. **Objective:** In this study, we compared the immunological properties of recombinant (r) Lol p 1 with those of the natural protein. **Method:** We cloned directly the gene encoding Lol p 1 from genomic DNA of ryegrass pollen. This gene was subcloned into the expression vector pMAL[®]-c and expressed as fusion protein. Subsequently, rLol p 1 was cleaved from maltose-binding protein using factor Xa. Using binding inhibition and proliferative assays, we assessed the immunological properties of the recombinant allergens. The capacity of rLol p 1 to trigger basophil histamine release and to elicit a skin reaction was also assessed and compared to those of its natural counterpart. **Results:** We found that the Lol p 1 gene has no introns since we amplified this gene directly from genomic DNA. We demonstrated that the binding sites of anti-Lol p 1 monoclonal antibody, specific human IgG and IgE antibody are well conserved on rLol p 1 as no difference in the binding inhibition profile was observed when using either natural or recombinant protein. At the T-cell level, rLol p 1 elicited a T-cell response in mice comparable to that observed with the natural protein. In addition, we demonstrated that the biological characteristics of rLol p 1 were comparable to those of the natural counterpart, in that rLol p 1 elicited a skin wheal reaction and induced basophil histamine release in grass-allergic patients only. **Conclusion:** The data indicate that natural Lol p 1 and rLol p 1 shared identical immunological and biological properties.

Key Words

Lol p 1
 Recombinant allergen
 Biological properties
 Pollen

Introduction

Allergens are usually extracted from natural sources, such as pollen, mites and animal danders. Sometimes, the use of these extracts is not suitable for diagnostic or therapeutic purposes because some natural allergens are rapidly

denatured during and after extraction [1–3]. Moreover it is possible that some allergens are missing, some exist in very low concentrations in extracts, and others are poorly extracted. Many studies have suggested that standardized allergen extracts or purified allergens instead of crude extracts could be used in both diagnosis and therapy to im-

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prove the management of allergic patients [4, 5]. Standardization of allergenic extracts could not be achieved without the availability of purified allergens. However, the purification of multiple allergic components from a given extract is a fastidious, sometimes impossible task.

In recent years, rapid progress has been made in relation to the molecular characterization of allergens by the application of recombinant DNA procedures. Thus, the cDNA of several proteins from different allergenic sources have been cloned, and the corresponding recombinant allergens have been produced by genetic engineering by either fusion or nonfusion techniques [6–12]. Cloning and expression of allergens with recombinant DNA have greatly facilitated the identification, characterization and analysis of various T- and B-cell epitopes [13, 14]. Recombinant allergens are also useful in the analysis of cross-reactivities between allergens, especially those from grass pollens [15, 16].

In this study we produce recombinant (r) Lol p 1 as a fusion protein. Lol p 1 is the major allergenic determinant of *Lolium perenne* pollen, one of the major species responsible for grass pollen allergy worldwide. This allergen is an abundant acidic glycoprotein with a molecular weight of 34 kD and a pI of 5.5 [17–19]. It is the major IgE-binding protein and approximately 90% of ryegrass-sensitive patients have had skin tests positive for Lol p 1 [20]. Moreover, elevated levels of Lol-p-1-specific IgE have been detected in the sera of up to 95% of grass-pollen-allergic individuals [20].

The gene encoding Lol p 1 was cloned into an expression vector to produce recombinant protein. Subsequently, binding inhibition of specific antibody, induction of T-cell proliferation and induction of basophil histamine release and wheal reaction in grass-sensitive patients were assessed using rLol p 1. These data were then compared to those obtained using natural (n) Lol p 1.

Materials and Methods

nLol p 1 and Monoclonal Antibodies

nLol p 1 was purified from commercial extracts (Omega Laboratories, Montréal, Canada) by an immunosorbent technique using anti-Lol-p-1 monoclonal antibody (mAb; 290A-167) fixed on solid phase as previously described. Briefly, concentrated crude ryegrass extracts were incubated overnight at 4°C with constant agitation with mAb-coated agarose beads. The bead suspension was then transferred into a column and extensively washed with phosphate-buffered saline, pH 7.2. The antigen was eluted using 3 M MgCl₂ in phosphate-buffered saline, pH 7.2. Hybridomas producing the anti-Lol-p-1 mAb 290A-167, 4F7, 8H5 and 1H5 were obtained from the fusion of Sp2/O-Ag14 cells and spleen cells of mice immunized with nLol p 1. All of these mAb were purified from ascitic fluids or cell culture supernatants by affinity chromatography on protein G (Gibco-BRL, Gaithersburg, Md., USA).

Direct Amplification of the Gene Encoding Lol p 1 by Polymerase Chain Reaction

Genomic DNA was extracted from nondefatted perennial ryegrass pollen according to the cetyltrimethylammonium bromide nucleic acid extraction procedure [21]. The coding sequence for the Lol p 1 protein was amplified using PCR with the following primers: 5'GTAA-GATATCTTCCTGGGCAGCGCATGGC3' and 5'GATTGGAT-CCTCACTTGGCCGAGTAGGAAG3'. These primers were designed so that 3' *Bam*HI and 5' *Eco*RV sites were available on the amplified product. Specific primers were diluted in PCR buffer containing 10 mM of dNTP. After an initial denaturation step at 94°C for 3 min, Taq polymerase (5 U) was added, and the DNA was amplified for 35 cycles on a thermal cycler as follows: denaturation at 92°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. The amplified products were purified by agarose gel electrophoresis, end-filled and ligated into the *Sma*I site of the pBluescript II KS- (Stratagene, La Jolla, Calif., USA). The cloned gene was sequenced using the T7 Sequencing kit (Pharmacia, Uppsala, Sweden).

Expression and Purification of rLol p 1 Protein in Escherichia coli

The complete gene encoding Lol p 1 was digested with *Eco*RV and *Bam*HI and subcloned into the pMAL-c vector (New England Bio-Labs, Beverly, Mass., USA) in frame with maltose-binding protein (MBP). After transformation of *E. coli* strain TB1 with the recombinant plasmid, the fusion protein was expressed and purified by affinity chromatography using amylose. The recombinant fusion protein was cleaved using factor Xa, and the resulting rLol p 1 was separated from MBP fragments using an amylose column.

Binding Inhibition of Human IgE and IgG Antibodies

The capacity of nLol p 1 or rLol p 1 to inhibit the binding of human serum IgE or IgG to Lol p 1 was assessed. nLol-p-1- or rLol-p-1-coated plates (100 ng/well) were incubated with diluted sera from grass-sensitive patients (1/4 for IgE and 1/10 for IgG analyses), alone or in the presence of various concentrations of nLol p 1 or rLol p 1 (0.001–10 µg/ml). After washes, ¹²⁵I-labeled antihuman IgE mAb (10⁵ cpm/well) or ¹²⁵I-labeled antihuman IgG (Caltag, San Francisco, Calif., USA) were added and incubated for 2 h at 37°C. After extensive washes, the bound radioactivity was measured using a gamma counter (LKB Wallac, Turku, Finland), and the percentage of inhibition was calculated as follows:

$$\left[1 - \frac{\text{cpm bound in the presence of inhibitor}}{\text{cpm bound in the absence of inhibitor}} \right] \times 100.$$

Binding Inhibition of Anti-Lol-p-1 mAb

Briefly, microtiter plates coated either with nLol p 1 or rLol p 1 were saturated with 3% bovine serum albumin in Tris-buffered saline. ¹²⁵I-labeled anti-Lol-p-1 mAb at a concentration corresponding to 50% of maximum binding were preincubated with various dilutions of natural allergen or rLol p 1 (0.002–20 µg/ml) for 1 h at 37°C. The mixture was added to the coated plates and incubated for 2 h at 37°C. After extensive washes, the bound radioactivity was measured and the percentage of inhibition was calculated as described above.

Skin Testing

A skin prick test was assessed in 6 grass-sensitive patients and 3 normal subjects using serial 10-fold dilutions of rLol p 1 or nLol p 1 (0.001–10 µg/ml) in saline. Positive (histamine phosphate 1 mg/ml)

and negative controls (saline) were also included. Fifteen minutes after administration of allergen, the mean diameter of the wheal reactions was measured.

Basophil Histamine Release Assays

The basophil histamine release (BHR) procedure was assessed as previously reported by our group [22]. Briefly, leukocytes containing basophils were isolated by dextran sedimentation. After extensive washes, the cells (4×10^6 cells/ml/tube) were incubated alone or with various concentrations (1 to 10^{-5} $\mu\text{g/ml}$) of nLol p 1 or rLol p 1 for 30 min at 37°C . The reaction was stopped by cooling, and the supernatants were recovered for histamine determination by a radioenzymatic assay. The percentage of histamine release was calculated as follows:

$$\text{BHR (\%)} = \frac{(A - B)}{T} \times 100,$$

where A = allergen-induced BHR, B = buffer-induced BHR and T = total histamine.

Mouse T-Cell Proliferation Assays

Mice were injected at the base of the tail with $100 \mu\text{g}$ of nLol p 1 without the use of adjuvant. T cells were obtained from the inguinal and mesenteric lymph nodes 8 days after the immunization of mice with $100 \mu\text{g}$ of nLol p 1. Irradiated antigen-presenting cells (2×10^6) were added to 1×10^5 T cells and incubated for 5 days with various concentrations of nLol p 1 or rLol p 1 (0.016 – $10 \mu\text{g/ml}$) in a final volume of $200 \mu\text{l/well}$. DNA synthesis was measured by adding $2 \mu\text{Ci}$ of [^3H]thymidine during the final 16 h. Cultures were harvested onto glass fiber filters with a cell harvester, and radioactivity was measured using a Microbeta counter (LKB Wallac).

Results

Expression and Purification of rLol p 1

Because the nucleotide sequence of the gene encoding Lol p 1 is known [7, 9], we designed primers to amplify this gene using PCR directly from genomic DNA. This finding indicated that the gene encoding Lol p 1 possessed no introns. The amplified DNA was then cloned into the BlueScript vector and sequenced. The nucleotide sequence of our clones corresponded exactly to the sequence of clone 1A obtained by Perez et al. [9]. The primers used for PCR amplification were designed to contain *Bam*HI and *Eco*RV restriction sites to facilitate insertion of the gene into the expression vector pMAL-c. With this vector, the cloned gene was inserted downstream from the *malE* gene, which encodes MBP fusion protein. This expression system, coupled with affinity purification using an amylose column yielded 17 – $20 \mu\text{g}$ of fusion protein per milliliter of *E. coli* culture. Using factor Xa, the recombinant protein was further cleaved to give rLol p 1 and MBP. The rLol p 1 and MBP were separated by a further passage on an amylose column with rLol p 1 eluted in the flowthrough. As shown in

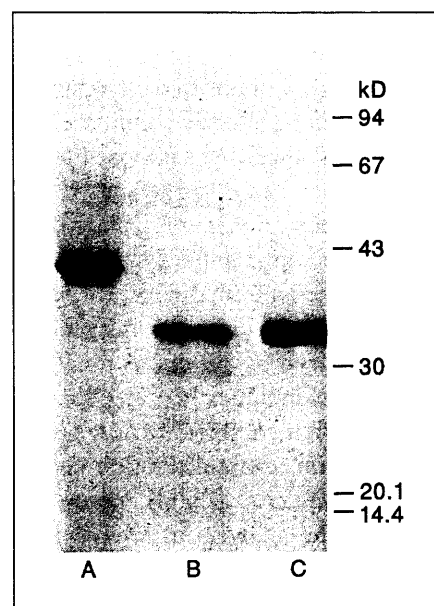


Fig. 1. Expression and purification of rLol p 1 as a fusion protein. Recombinant allergen was affinity-purified from lysates of bacteria as described in Materials and Methods. Purified proteins were characterized by 12% SDS-PAGE, and the gel was stained with Coomassie blue. Lane A represents the affinity-purified fusion protein, and lane B represents purified rLol p 1 after cleavage with factor Xa. The molecular weight of rLol p 1 was compared with that of nLol p 1 (lane C). Molecular size markers (kD) are indicated on the right.

figure 1, rLol p 1 with a molecular weight of 34 kD has the same electrophoretic mobility as nLol p 1.

Immunological Properties of rLol p 1

The use of rLol p 1 for both diagnostic and therapeutic purposes requires that the recombinant and natural allergens share immunological properties. Using binding inhibition assays we further compared antigenic and allergenic features of both rLol p 1 and nLol p 1. In these assays, nLol p 1 was used as solid phase and anti-Lol-p-1 mAb or a pool of sera ($n = 6$) from grass-sensitive patients were added alone or along with various concentrations of either nLol p 1 or rLol p 1.

Figure 2 displays the inhibition of anti-Lol-p-1 mAb to nLol p 1 by various concentrations of either nLol p 1 or rLol p 1. These mAb were directed against different epitopes of the molecule, and 2 of them (290A-167 and 4F7) recognized IgE-binding epitopes. The binding of these 4 mAb to solid-phase-bound nLol p 1 was inhibited by increasing the amounts of either nLol p 1 or rLol p 1, and no difference was observed in the binding inhibition capacity of these molecules.

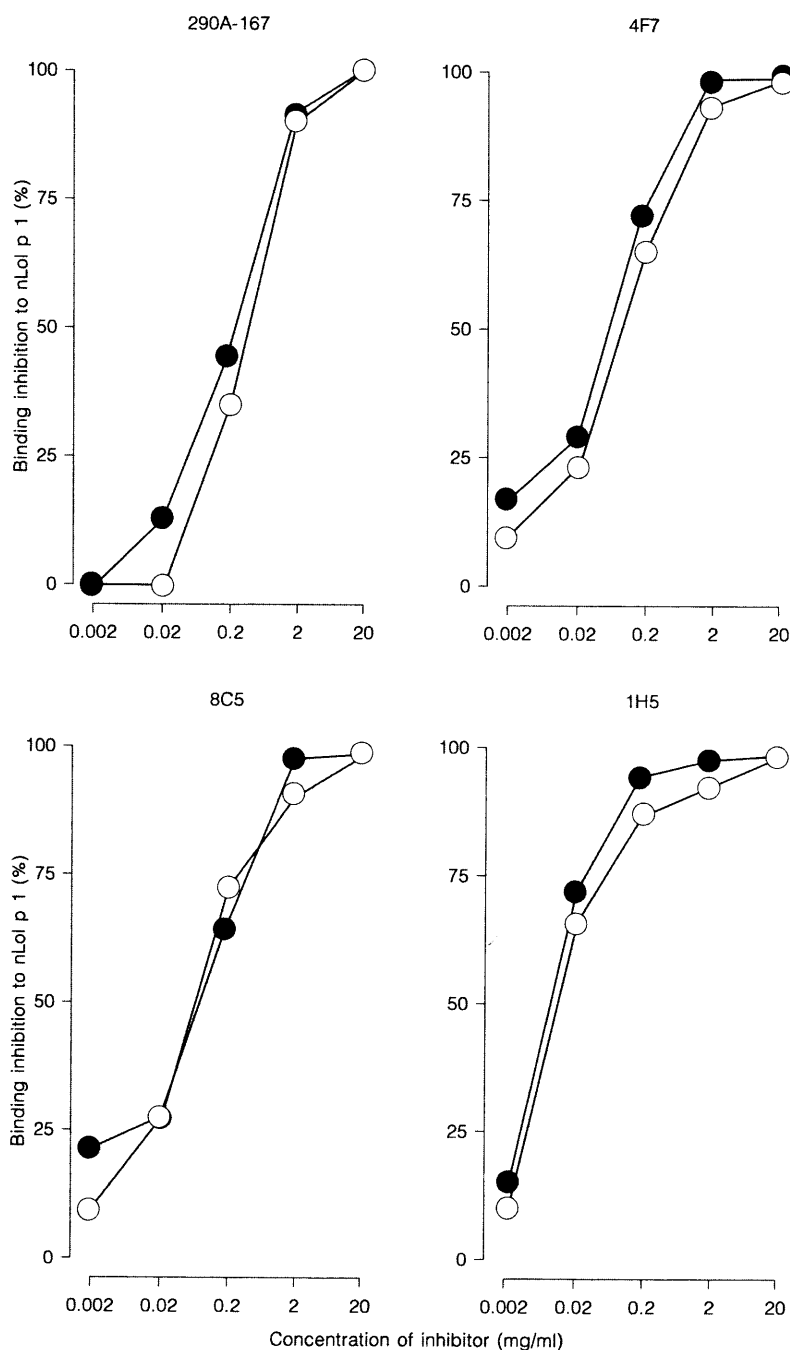


Fig. 2. Immunological properties of rLol p 1. Binding inhibition of 4 anti-Lol-p-1 mAb (290A-167, 4F7, 8C5 and 1H5) to nLol p 1 by various concentrations of either nLol p 1 (○) or rLol p 1 (●).

The antigenic and allergenic similarities between nLol p 1 and rLol p 1 were further demonstrated in the binding inhibition of either human IgG (fig. 3a) or human IgE (fig. 3b) on nLol p 1. In these two assays, both rLol p 1 and nLol p 1 fully inhibited the binding of IgE or IgG to nLol p 1. Furthermore the profile of inhibition of rLol p 1 is comparable to that obtained with nLol p 1. No inhibition of ei-

ther IgE or IgG was observed when birch extracts were used as control. Identical results in binding inhibition studies were obtained when rLol p 1, instead of nLol p 1, was used in the solid phase. Taken together, the binding inhibition data indicate that the epitopes recognized by our mAb, human IgE and human IgG are properly displayed on rLol p 1.

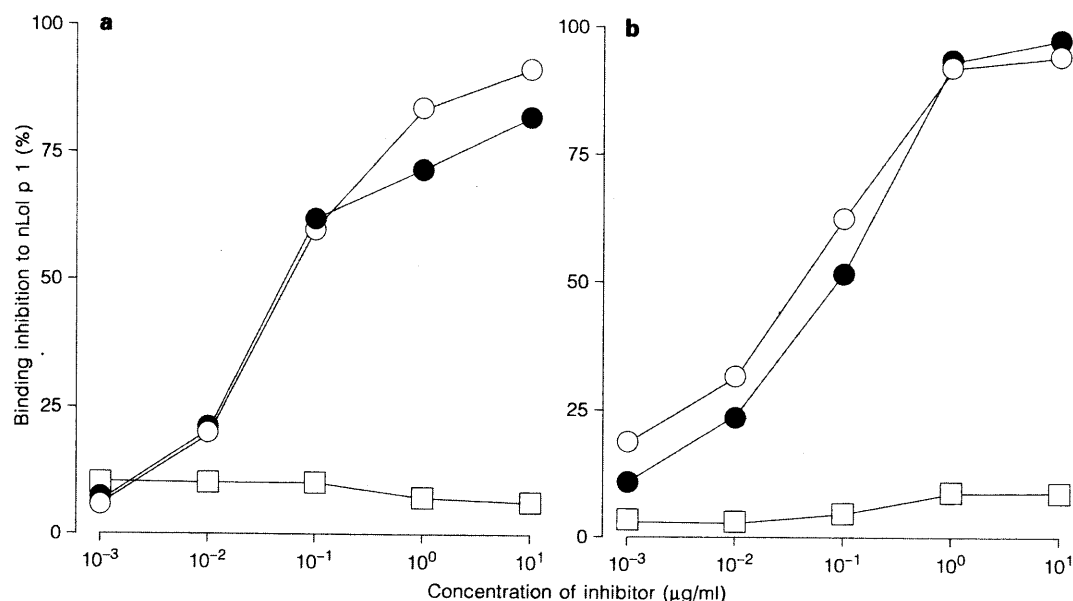


Fig. 3. Immunological properties of rLol p 1. Inhibition of the binding of specific human IgG (a) or IgE (b) to nLol p 1. Specific human antibodies were inhibited by either nLol p 1 (○) or rLol p 1 (●) in a dose-dependent fashion. Birch allergen (□) was used as control.

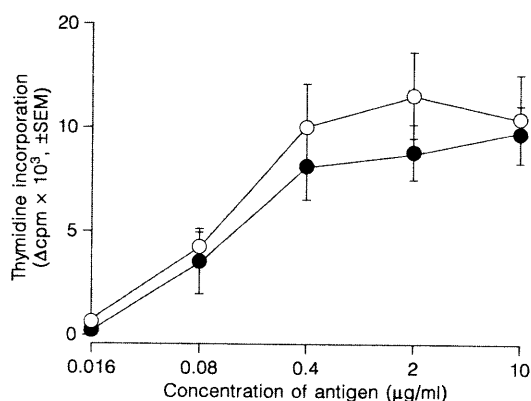


Fig. 4. Comparison of T-cell reactivities to nLol p 1 (○) or rLol p 1 (●). Lymph node T cells from mice immunized with natural protein were cultured (2×10^4 /well) for 6 days with irradiated autologous antigen-presenting cells (10^5 /well) in the presence or absence of antigen. Each point represents the mean of 3 different experiments.

We also compared nLol p 1 and rLol p 1 at the T-cell level (fig. 4). Lymph node mouse T cells were tested for their ability to proliferate when stimulated with either nLol p 1 or rLol p 1. T cells isolated from mice immunized with nLol p 1 were incubated with different concentrations of antigen in the presence of irradiated autologous antigen-presenting cells. The results were expressed as Δ cpm where the incorporation of [^{3} H]thymidine into DNA by T cells in the presence of either nLol p 1 or rLol p 1 was subtracted by the incorporation in the absence of protein. T cells from mice primed with nLol p 1 proliferated in a dose-dependent fashion to both nLol p 1 and rLol p 1. No significant difference was observed in the pattern of reactivity when using nLol p 1 or rLol p 1 to stimulate T-cell proliferation. These data suggested that T-cell epitopes are well conserved on rLol p 1.

Biological Activities of rLol p 1

Binding inhibition assays and T-cell proliferation assays have demonstrated that rLol p 1 is immunologically comparable to the natural protein. We further analyzed the biological activities of rLol p 1 to elicit skin reactivity and to induce histamine release from basophils of grass-sensitive patients.

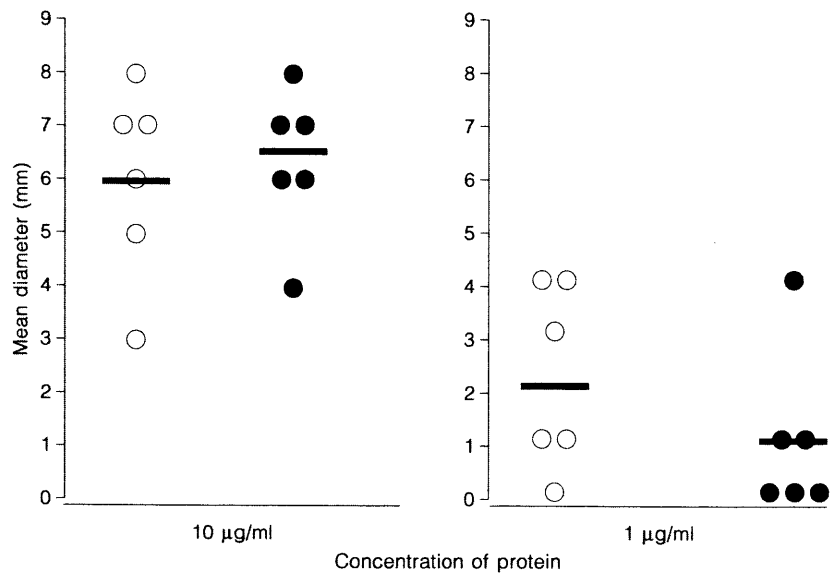


Fig. 5. Biological properties of rLol p 1. Wheal reactions in grass-sensitive patients elicited by injection of 1 and 10 µg/ml of nLol p 1 (○) or rLol p 1 (●).

Skin prick tests were performed on the forearm of atopic and nonatopic individuals using different concentrations of nLol p 1 or rLol p 1. Significant wheal reactions were observed with concentrations of 10 µg/ml or 1 µg/ml only. Indeed, wheal reactions induced by 10 µg/ml of nLol p 1 or nLol p 1 were 6.0 and 6.3 mm of diameter, respectively (fig. 5). No significant difference was observed when 1 µg/ml of either nLol p 1 or rLol p 1 was injected. Furthermore no reaction was elicited by either molecule in nonatopic patients (data not shown). Thus, these data showed no difference between nLol p 1 and rLol p 1 in their capacity to elicit skin reactivity.

Finally, the ability of natural and recombinant proteins to release histamine from basophils of allergic or control subjects was also assessed. Different concentrations of Lol p 1, ranging from 10^{-5} to 1 µg/ml were used to trigger basophils from 4 grass-sensitive patients. As shown in figure 6, the profile of histamine released by rLol p 1 is comparable to that obtained with nLol p 1 for each concentration. No histamine was released from basophils of nonallergic subjects upon either nLol p 1 or rLol p 1 challenge (data not shown). The results obtained with BHR assays strengthened those obtained with binding inhibition assays, indicating that IgE-binding epitopes found on nLol p 1 are also present on the recombinant allergen.

Discussion

Purification and characterization of allergens from crude extracts involve time-consuming techniques, such as gel chromatography, isoelectric focusing, affinity chromatography and preparation of mAb. Furthermore during the purification process, allergenic components could be altered or lost. To overcome these problems, recombinant DNA technology could provide unlimited amounts of well-defined cloned allergens.

The use of recombinant allergens for any diagnostic or therapeutic purpose further requires that these recombinant allergens meet some important criteria, such as purity and stability, and their immunological and biological properties must be the same as their natural counterparts. In fact, the IgE-binding capacity of recombinant allergens is sometimes less than that of the corresponding natural allergens. Clearly, this difference in binding affinity may be due to differences in the folding of recombinant allergens when they were expressed as fusion proteins using some expression vectors such as the glutathione-S transferase system [23, 24]. We use the pMAL expression system to produce fusion proteins. This system is attractive for several reasons: proteins can be expressed at high levels [25], the fusion protein can be easily purified from the cellular content by affinity chromatography, and it has a polylinker containing the sequence coding for the factor Xa protease recognition site permitting a separation of the MBP from recom-

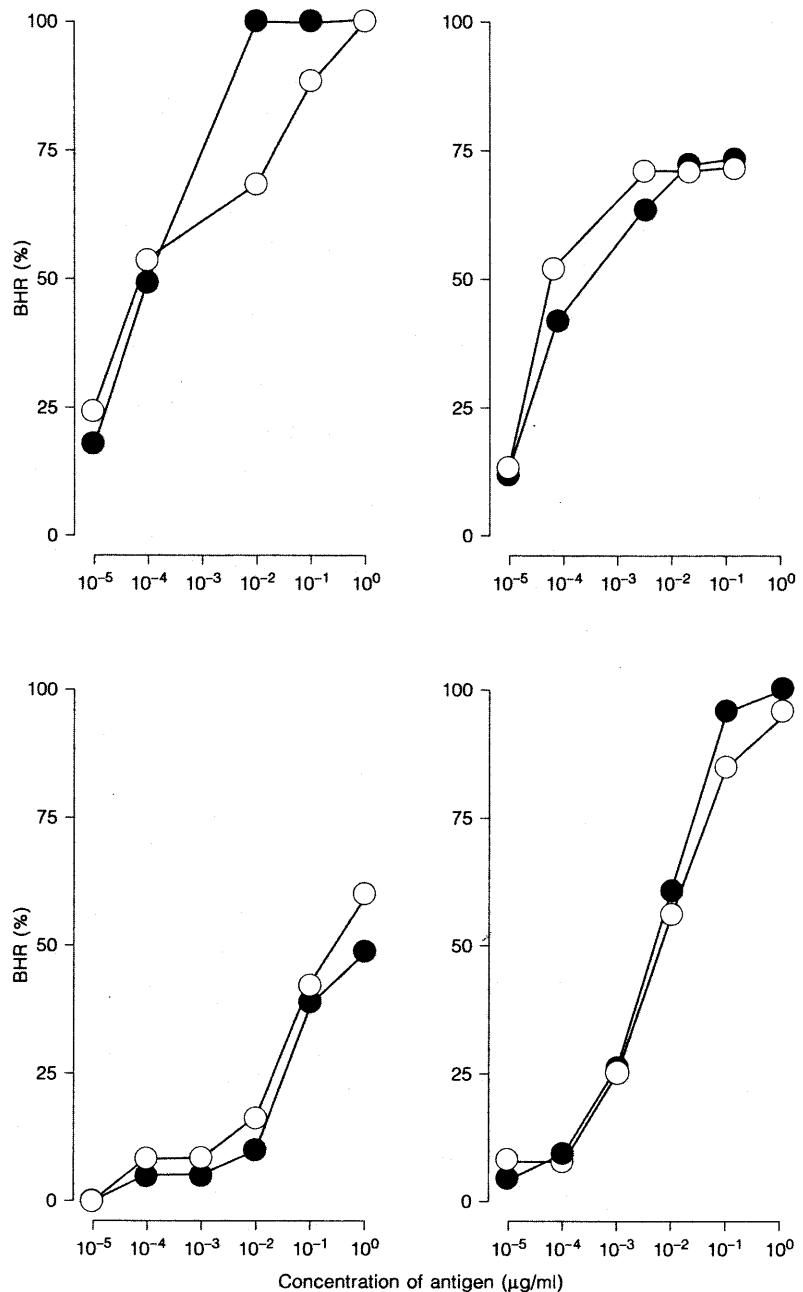


Fig. 6. Biological properties of rLol p 1. Histamine release from 4 grass-sensitive patients triggered by different concentrations of nLol p 1 (○) or rLol p 1 (●).

binant protein. Moreover, with the appropriate vector, the fusion can be produced in the bacterial periplasm instead of in the cytoplasm. This is interesting, especially when the protein of interest does not fold properly in the *E. coli* cytoplasm, as sometimes occurs with proteins containing disulfide bonds. However, for allergens which contain carbohydrate residues, the expression of recombinant proteins in prokaryotes might not be the best choice. In such a case, a

eukaryote expression system should be considered. Even though Lol p 1 is a glycoprotein, it was demonstrated that most IgE antibodies react against the protein core and few patients recognized the carbohydrate moieties in group I allergens from grass pollens [26]. Thus, the use of bacteria remains a good choice to produce rLol p 1.

Using PCR, we amplified the gene encoding Lol p 1 directly from genomic DNA, indicating that there are no in-

trons. The gene was then cloned into the pMAL-c expression vector to produce rLol p I. Although the fusion protein is produced in the cytoplasm, it appears that the rLol p I has the appropriate folding since its immunological and biological properties were similar to those of the natural allergen. In fact, comparison of IgE- and IgG-binding reactivities of natural and recombinant proteins by competitive immunoassays showed no significant differences. It is worth noting that these assays could also detect conformational epitopes since they did not denature the protein as Western blot analysis did.

Proliferation assays, skin tests and BHR confirmed the results obtained with immunoassays and indicated that the rLol p I produced with the pMAL expression vector could be suitable for diagnostic tests and possibly for treatment. Finally, recombinant DNA technology offers us the possibility to produce rLol p I for in vitro and in vivo tests. Moreover, this technology should be useful in studies of allergen T- and B-cell epitopes. The availability of these epitopes may be useful in elucidating the mechanisms of IgE regulation and designing more efficient diagnostic and therapeutic tools.

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