

Oral Immunization with a Recombinant Major Grass Pollen Allergen Induces Blocking Antibodies in Mice

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Key Words

Allergy · Grass pollen · Immunotherapy · Lol p 5 ·
Rye grass

Abstract

Background: Immunotherapy for the treatment of pollen allergies traditionally involves a series of parenteral injections of a crude pollen extract. Successful application of this treatment results in the development of systemic tolerance to the sensitizing allergens, including the induction of blocking antibodies. **Objective:** We sought to investigate whether oral immunization with a recombinant pollen allergen could induce a systemic immune response, and the production of systemic blocking antibodies in mice. **Methods:** C57BL/10 mice were orally administered rLol p 5 or sodium chloride solution via gavage. **Results:** We report that the oral administration of rLol p 5 induced a systemic immune response, including the induction of both blocking and interspecific cross-reactive antibodies. **Conclusion:** Our results suggest that oral administration of a major grass pollen allergen can induce the development of a systemic immune response including the production of systemic blocking and cross-reactive antibodies, a response that may offer immunological protection upon subsequent allergen exposure.

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Introduction

The immunotherapeutic treatment of inhalant allergies traditionally involves a series of parenteral injections of a crude allergen extract, and has been used as such since 1911 [1]. Known as hyposensitization, since it aims to reduce patient sensitivity to the causative allergen, this form of immunotherapy has been clinically shown to be effective for atopic individuals sensitized against inhalant allergens, including grass pollen [2–4]. Immunotherapeutic reagents delivered via the parenteral route often induce systemic tolerance, including the induction of blocking antibodies, specific for the sensitizing allergens. Alternative routes for the delivery of immunotherapeutic reagents, such as the oral, nasal, bronchial and sublingual routes, are currently being investigated for improved patient compliance and reduced risk of a systemic anaphylactic response [5]. The oral administration of protein allergens has been demonstrated for the experimental treatment of house dust mite, birch and ragweed pollen allergy [6–9]. While delivering the immunotherapeutic agents directly to the gut mucosa in enteric coated capsules, these studies demonstrated oral immunotherapy to be simpler and easier to administer than conventional hyposensitization treatment [6–9]. Furthermore, these studies also showed that oral administration of pollen allergy immunotherapeutic reagents induced systemic

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and mucosal immune responses, and resulted in reduced allergic symptoms [6, 7, 9]. However, one of the main limitations of the oral route of antigen administration is that doses much greater than that administered via the subcutaneous route may be required to produce the desired clinical effects [10]. In this study we have used mice to investigate whether oral delivery of a highly purified recombinant form of the major rye grass pollen allergen, Lol p 5, could induce systemic antibodies able to block human IgE binding to this allergen and recognize homologous allergens from related grasses.

The levels of allergen-specific serum IgG and IgE antibodies induced upon oral administration of rLol p 5 and sodium chloride were compared. The capacity of these antibodies to inhibit human IgE binding to rLol p 5, and to cross-react with proteins from natural grass pollen extracts, was also investigated. The results suggest that the oral route of immunization with a recombinant pollen allergen can induce a systemic immune response, including the production of systemic blocking and cross-reactive antibodies in mice. The oral delivery of immunotherapeutic reagents may be an alternative route of immunization for improved efficacy of the allergen-specific immunotherapeutic treatment of allergic individuals.

Materials and Methods

Expression and Purification of Recombinant Lol p 5

Recombinant Lol p 5 (rLol p 5) was expressed as a fusion protein with an amino-terminal, six-residue histidine tag within the vector pQE31 (Qiagen, Hilden, Germany) in *Escherichia coli* cells BL21 (Stratagene, La Jolla, Calif., USA). The fusion protein was induced and purified according to the manufacturers' guidelines. Briefly, 500-ml cultures were seeded with fresh 10-ml cultures of the appropriate plasmids in BL21 cells, and grown at 37°C for 4 h. Expression of the fusion proteins was induced by the addition of 1 mM isopropyl thio-galactoside. After overnight incubation at 37°C, cells were harvested by centrifugation (3,840 g, 10 min) and resuspended in 20 ml of lysis buffer (8 M urea, 50 mM sodium dihydrogen orthophosphate, 10 mM tris-hydroxy-methane, 100 mM sodium chloride, pH 8.0). After gentle rocking on ice for 1 h, the preparation was centrifuged at 17,000 g for 5 min and the cellular pellet discarded. Two milliliters of washed Talon metal affinity resin (Clontech, Palo Alto, Calif., USA) was added to the supernatant with further gentle rocking on ice for 20 min. After washing three times with 20 ml of lysis buffer, the resin with bound sample was poured into a 1-ml disposable, polypropylene column (Qiagen). After formation of the resin column by gravity, the column was washed with three column volumes of lysis buffer, with a final high-stringency wash with wash buffer (8 M urea, 50 mM sodium dihydrogen orthophosphate, 100 mM sodium chloride, pH 7.0). Purified His-tagged proteins were eluted in 4 ml of elution buffer (8 M urea, 50 mM sodium dihydrogen orthophosphate, 20 mM morpholino-ethanesulfonic acid, 100 mM sodium chloride, pH 5.0) before dialysis against four changes of 0.05% sodium chloride in 2-

liter volumes. Proteins were dried down using a Speedvac (Savant, USA) and quantified using the Bradford method [11], with purity and concentration confirmed by SDS-PAGE [12] and Coomassie staining.

Animals

Fourteen-week-old C57BL/10 mice were used for these experiments. The mice were housed in the Department of Zoology Animal House, University of Melbourne. The mice were maintained on a diet of Barastoc G. R. 2 rat and mouse breeder rations (Ridley Agri-products, Pakenham, Australia). The *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (1989), published by the National Health and Medical Research Council, in conjunction with the Department of Agriculture was followed during the care of the experimental animals used in these trials.

Immunization Regime and Blood Sample Preparation

Ten mice were separated into two groups of 5 mice each. One group received 100 µg of rLol p 5 in a volume of 100 µl of 0.85% sodium chloride, while the second group received 100 µl of 0.85% sodium chloride. All protein or sodium chloride samples were delivered to the mice by gavage. Mice received rLol p 5 or sodium chloride solution on days 0, 7, 21 and 56. Blood samples were collected every 7 days throughout the course of the trials, and on days 0, 7, 21 and 56, bleeding was conducted prior to the administration of the protein or saline solutions. Serum was separated from the cellular component of the blood samples by centrifugation (at 10,000 g, 5 min) and stored at -20°C before use.

Direct ELISA Assays

Ninety-six-well Microton ELISA plates (Greiner, Germany) were used in all assays. Optimum serum and secondary antibody concentrations were determined by titration assays prior to use. Duplicate direct ELISAs for the measurement of specific IgG, IgG1, IgG2a and IgE were carried out on the sera of individual mice. For all direct ELISAs, wells were coated with 50 µl of 2 µg/ml rLol p 5 in PBS (0.15 M sodium chloride, 3.6 mM sodium dihydrogen orthophosphate, 6.7 mM disodium hydrogen orthophosphate) for 4 h at room temperature. After washing twice with PBST (PBS with 0.05% Tween 20) and three times with PBS, wells were blocked with 400 µl of serum dilution buffer [3% bovine serum albumin (BSA), 0.02% sodium azide in PBS] or 1% BSA in PBST overnight at 4°C. Wells were washed as before, followed by the addition of 50 µl/well of serum samples diluted in serum dilution buffer (for detection of IgG, IgG1 and IgG2a samples were diluted 1:2,000; for IgE, 1:20). Following 2 h of incubation at room temperature (or overnight at 4°C), serum samples were removed, before washing as above. Secondary antibodies for the detection of murine IgG [rabbit, anti-mouse IgG (used at 1:7,500 dilution; Promega, Madison, Wisc., USA)] and IgG1 or IgG2a [rabbit, anti-mouse IgG1 and IgG2a (used at 1:2,000 dilution; ICN Biomedicals, Costa Mesa, Calif., USA)] were conjugated with alkaline phosphatase. Goat, anti-mouse IgE (used at 1:1,000 dilution) was supplied unlabeled – this antibody was detected using alkaline phosphatase conjugated donkey anti-goat IgG (used at 1:2,500 dilution; ICN Biomedicals). Fifty microliters of the appropriate secondary antibody, diluted in serum dilution buffer, was added to each well. After 2 h of incubation at room temperature and washing as above, the alkaline phosphatase label was detected by incubation with 50 µl/well of 1 µg/ml *p*-nitrophenyl phosphate in alkaline phosphatase buffer (100 mM tris-hydroxymethane, 100 mM

sodium chloride, 50 mM magnesium chloride, pH 9.5). Plates were incubated at room temperature before reading at 405 nm in a Spectracount plate reader (Packard Instrument Company, Illinois, USA). Data for both rLol p 5- or sodium-chloride-immunized mice are reported as the mean with standard error, calculated from measurements of antibodies within sera collected from individual mice.

Inhibition ELISA Assays

For inhibition ELISA assays, wells were coated with 50 µl of 200 ng/ml rLol p 5 in PBS for 4 h at room temperature. After washing and blocking as described for direct ELISAs, 50 µl/well of pooled sera collected from the rLol p 5-immunized mice on day 70 was added. Pooled serum dilutions of 1:10, 1:20, 1:50 or 1:100 prepared in serum dilution buffer were used. After overnight incubation at 4°C and another series of washes, 50 µl of pooled sera from 10 grass-pollen-allergic patients diluted 1:5 in serum dilution buffer was added per well, and the plate incubated at room temperature for 4 h. After washing as above, bound human IgE antibodies were detected with 50 µl of a 1:1,000 dilution of alkaline-phosphatase-conjugated goat anti-human IgE antibodies (Sigma, St. Louis, Mo., USA). Detection was carried out as described for direct ELISAs. Inhibition of IgE binding is expressed as a percentage of the inhibition achieved by preincubation with BSA. Assays were performed in triplicate using pooled sera collected from the mice on day 70 and thus the data are reported as the mean with standard error.

Detection of Antibodies That Block Human IgE Binding to rLol p 5

Approximately 10, 50 and 100 ng of purified rLol p 5 were separated by 10% SDS-PAGE [12] and blotted onto PVDF by the method described above. Two identical blots were prepared and blocked in 10% milk powder in PBS for 1 h at room temperature with shaking. After washing twice in PBST and twice in PBS for 10 min each, one blot was incubated with sera collected from rLol p 5-immunized mice on day 70, pooled and diluted to 1:10 in sera dilution buffer. The second identical blot was incubated only in the serum dilution buffer, which contained 1% BSA. Following overnight incubation and washing, both blots were incubated at room temperature overnight with a 1:5 dilution of sera, pooled from 10 grass-pollen-allergic patients. Following another series of washes, the blots were incubated with alkaline-phosphatase-conjugated goat anti-human IgE antibodies (Sigma) diluted 1:1,000 in serum dilution buffer. After 2 h of shaking at room temperature and another series of washes, bound alkaline-phosphatase-conjugated antibodies were detected by incubation in 0.4 mM nitroblue tetrazolium (Boehringer, Mannheim, Germany), 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (Astral Scientific, Gympie, Australia) in alkaline phosphatase buffer. Color development was halted by incubation in 0.1 M ethylene diamine-tetraacetic acid.

Detection of Interspecific Cross-Reactive Antibodies

Proteins were extracted from the pollen of six *Pooideae* grass species – *Festuca* sp., *Holcus lanatus*, *Phalaris arundinacea*, *Phleum pratense*, *Poa pratensis* and *Lolium perenne*. Proteins were extracted by grinding in 500 µl of PBS containing 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 10,000 *g* for 5 min, the concentration of proteins in the supernatant was measured by the method of Bradford [11]. For Western blotting, 20 µg of pollen proteins from each species were separated by SDS-PAGE (15%) [12] on a mini-Protein II protein gel system in a buffer of 200 mM glycine, 25 mM tris-

hydroxymethane, 0.1% sodium dodecyl sulfate (Biorad, Hercules, USA). Two identical blots were prepared and electroblotted onto PVDF membranes (Osmonics, Westborough, USA) using a mini-Protein II electroblotting system (Biorad) in a buffer of 200 mM glycine, 25 mM tris-hydroxy-methane and 20% methanol at 100 V for 70 min. After drying, the blots were blocked in 10% milk powder in PBS for 1 h at room temperature with shaking. After washing twice in PBST and twice in PBS for 10 min each, the blots were incubated in 1:200 diluted, pooled sera collected prior to initial immunization and on day 70 from mice immunized with rLol p 5. After overnight shaking at room temperature, the blots were washed as above and incubated with an alkaline-phosphatase-conjugated goat anti-mouse IgG antibody (Promega) diluted 1:7,500 in serum dilution buffer. After 2 h of shaking at room temperature, the blots were again washed as above, and rinsed in alkaline phosphatase buffer before incubation in 0.4 mM nitroblue tetrazolium (Boehringer), 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (Astral Scientific) prepared in alkaline phosphatase buffer. Color development was halted by incubation in 0.1 M ethylenediaminetetraacetic acid.

Results

Oral Immunization with rLol p 5 Leads to Systemic Antibody Production

All mice immunized with rLol p 5 showed an increase in anti-rLol p 5 IgG and IgE antibody levels in serum samples up to day 28 (fig. 1a, b). From day 28 to 56, these mice showed a fall in levels of serum, specific IgG and IgE antibodies (fig. 1a, b). Following administration of the booster dose on day 56, the levels of anti-rLol p 5 IgG and IgE antibodies detected in the sera collected on day 70 from the rLol p 5-immunized mice were higher than any other day tested (fig. 1a, b). As can be seen from figure 1, sera collected from mice orally administered sodium chloride solution showed insignificant absorbency readings at 405 nm, as compared to mice immunized with rLol p 5.

All mice immunized with rLol p 5 showed detectable levels of anti-rLol p 5 IgG1 antibodies but low levels of specific IgG2a antibodies in sera collected from mice on days 28, 56 and 70 (fig. 2). In the serum samples collected from the mice on days 28 and 56, considerable variability was recorded (fig. 2). The highest levels of both anti-rLol p 5-specific IgG1 and IgG2a antibodies were detected in the samples collected on day 70 (fig. 2).

Mice Immunized with rLol p 5 Produced Antibodies that Blocked Human IgE Binding to rLol p 5

Antibodies in sera collected on day 70 from mice orally immunized with rLol p 5 were tested over a range of sera dilutions for the ability to inhibit human IgE binding to rLol p 5. The pooled murine sera samples inhibited human IgE binding to rLol p 5 between 68.4% (at a 1:10

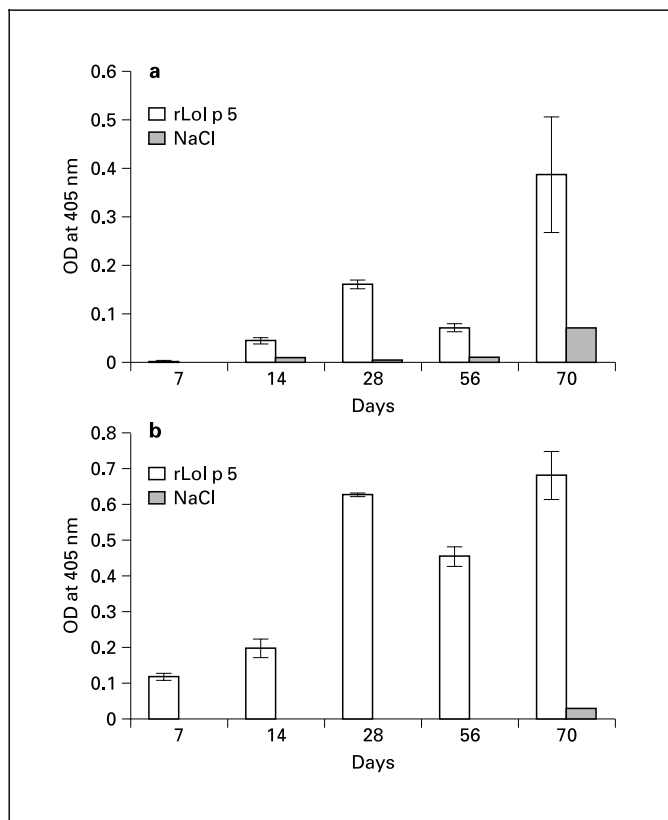


Fig. 1. Levels of anti-rLol p 5 IgG (a) and IgE (b) antibodies detected in diluted sera collected on days 7, 14, 28, 56 and 70 from mice orally immunized with rLol p 5 (rLol p 5) or sodium chloride solution (NaCl). Data points represent OD at 405 nm above those measured for preimmune (day 0) sera. Standard error bars indicate the level of variation between individual mice for each time point.

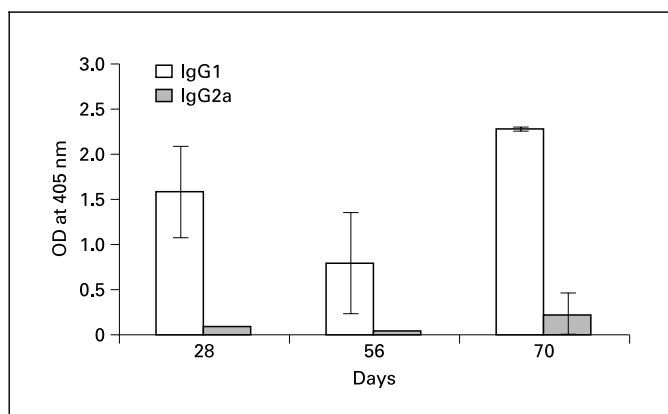


Fig. 2. Levels of anti-rLol p 5 IgG1 and IgG2a antibodies detected in diluted sera collected on days 28, 56 and 70 from mice orally immunized with rLol p 5. Data points represent OD at 405 nm above those measured for preimmune (day 0) sera. Standard error bars indicate the level of variation between individual mice for each time point.

dilution) and 43% (at a 1:100 dilution) (table 1). The ability of murine antibodies to block human IgE binding to rLol p 5 was also visualized using a western blot inhibition assay. Figure 3 shows that human IgE antibodies from the sera of grass pollen allergic patients bind strongly to rLol p 5 after prior incubation with BSA. However, incubation of an identical western blot with pooled murine sera prior to immunoscreening with allergic patient sera clearly inhibited human IgE antibody binding to the recombinant allergen (fig. 3).

Oral Immunization with rLol p 5 Induces Antibodies that Cross-React with Proteins from the Pollen Extracts of Other Grasses

Western blots of the pollen extracts from 6 *Pooideae* grass species were incubated with the pooled sera collected from rLol p 5 immunized mice prior to immuniza-

Table 1. Percentage inhibition of human IgE antibody binding to rLol p 5 by prior incubation with the pooled sera collected on day 70 from mice orally immunized with rLol p 5

Serum dilution	Percentage inhibition
1:10	68.4
1:20	60.5
1:50	47.5
1:100	43.0

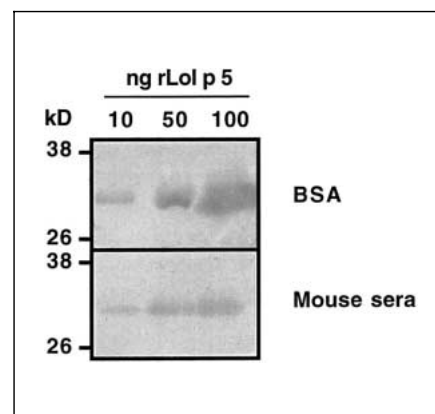


Fig. 3. Western blot immunoscreening of rLol p 5 (10, 50, and 100 ng) with human IgE antibodies after prior incubation with BSA and the pooled sera collected on day 70 from mice orally immunized with rLol p 5.

tion (preimmune) and on day 70. Incubation of the Western blots with preimmune sera showed the presence of murine IgG antibodies that bound to unidentified proteins in the range of 40–43 kD in 5 of the 6 grass pollen extracts tested. On the western blot incubated with sera collected from rLol p 5-immunized mice on day 70, IgG antibodies also bound to proteins of 28–33 kD (the approximate size of group 5 grass pollen allergens) in pollen extracts from all 6 species, indicating that antibodies able to recognize group 5 homologous proteins from related grasses were induced by the oral administration of Lol p 5 (fig. 4).

Discussion

More than 90% of grass-pollen-allergic patients are sensitized against Lol p 5, the group 5 pollen allergen from *L. perenne*. Subcutaneously administered hyposensitization immunotherapy has been demonstrated to be effective for grass pollen allergy [2–4]. However, this form of allergy immunotherapy can lead to the development of anaphylaxis, a life-threatening, systemic allergic response [13]. The oral administration of encapsulated pollen allergens, while inducing both systemic and mucosal immune responses in human trials, has been shown to be simpler and easier to administer, and effective in reducing allergy symptoms [7–9]. In the present study, we have demonstrated that the oral immunization of naïve mice with soluble rLol p 5 can induce a systemic immune response,

with the production of blocking and interspecific cross-reactive antibodies.

Although the mechanisms underlying allergy immunotherapy are not precisely known, a number of studies have demonstrated that the induction of ‘blocking’ antibodies may be important for its successful application [14, 15]. While such blocking antibodies have been found to belong primarily to the IgG class, their role in the successful application of immunotherapy in pollen-allergic patients remains inconclusive [16, 17]. However, the beneficial effects of blocking antibody induction as a result of immunotherapeutic treatment has been reported using a Bet v 1 human IgG monoclonal antibody established from a hyposensitized patient [18]. These antibodies were shown to strongly suppress human IgE binding to the major birch pollen allergen Bet v 1 [18]. Furthermore, specific IgG antibodies have also been shown to suppress IgE-facilitated release of mediators from effector cells and to inhibit serum-IgE-facilitated antigen presentation, thereby reducing T cell proliferation, cytokine production and the severity of immediate symptoms [18–22]. The subcutaneous immunization of mice with recombinant timothy grass pollen allergens has been shown to induce the production of specific IgG1 antibodies that were subsequently shown to strongly inhibit human IgE antibody binding to these allergens and to prevent allergen-induced, histamine release from basophils [20]. In the present study, we demonstrated that the oral administration of a recombinant pollen allergen could similarly induce the production of allergen-specific IgG1 antibodies. Furthermore, antibodies induced following the oral immunization protocol were shown to possess the ability to block the interaction between human IgE antibodies and rLol p 5. Titration of the blocking activity of the sera of rLol p 5-immunized mice demonstrated the efficacy of the antibodies to block human IgE antibody recognition of rLol p 5 to a dilution of 1/100 of the pooled sera.

An important property of an immunotherapeutic reagent for the treatment of grass pollen allergy would be its ability to induce systemic antibodies capable of recognizing homologous allergens from other grass species [20]. Upon subcutaneous immunization of mice, an immunogenic fragment of the major birch pollen allergen, Bet v 1, has been demonstrated to induce the production of IgG antibodies that could cross-react with Bet v 1 and homologous allergens from other plant species, and inhibit the interaction between these allergens and human IgE antibodies [23]. Due to the demonstrated IgE cross-reactivity of group 5 grass pollen allergens [24], an immunization protocol that induces interspecific cross-reactive anti-

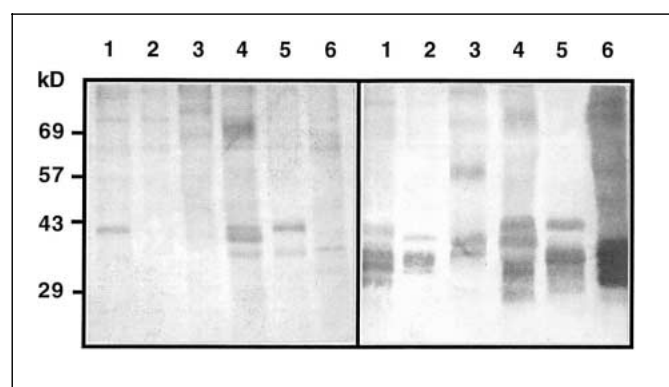


Fig. 4. Detection of interspecific cross-reactive IgG antibodies in pooled sera collected prior to immunization (left panel) and on day 70 (right panel) from mice orally immunized with rLol p 5. Pollen extracts from *Festuca* sp. (1), *Holcus lanatus* (2), *Phalaris arundinaceae* (3), *Phleum pratense* (4), *Poa pratensis* (5), and *Lolium perenne* (6) were tested.

bodies upon administration of a purified group 5 allergen could offer the immunotherapy recipient immunological protection from homologous allergens. In the present study, it was shown that oral immunization of naïve mice with rLol p 5 led to the production of IgG antibodies able to bind to Lol p 5 and proteins from the pollen of related grasses. Induction of these antibodies, which recognized proteins of the size corresponding to group 5 grass pollen allergens in extracts from 6 *Pooideae* grasses, indicates that the oral immunization of mice with rLol p 5 could induce the production of antibodies capable of cross-reacting with group 5 homologous proteins from rye grass and also related grass species.

In conclusion, we have shown that oral administration of a major pollen allergen to naïve mice can elicit a systemic immune response, including the production of

blocking and interspecific cross-reactive antibodies. These results suggest that oral immunization with a major pollen allergen may be useful for the induction of protective immune responses for the treatment of pollen hypersensitivity, and may aid in the development of alternative immunotherapeutic protocols for the treatment of pollen allergy.

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