

Sensitization to *Lolium multiflorum* Grass Pollen in Pollinosis Patients: Evaluation of Allergenic Fractions Recognized by Specific IgE Antibodies

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Key Words

Allergic rhinoconjunctivitis · Grass pollen allergy · Immunoglobulin E · *Lolium multiflorum* · *Lolium perenne*

Abstract

Background: *Lolium multiflorum* (Lm) pollen allergens are the major causative agents for rhinoconjunctivitis in Southern Brazil. There have been no studies about the sensitization and allergenic cross-reactivity between Lm and other grass pollens. We evaluated the sensitization of Brazilian pollinosis patients to Lm pollen allergens through skin prick test (SPT) and immunoassays (ELISA and immunoblot). **Methods:** Serum samples from 60 patients with pollinosis and positive SPT to grass pollen

extracts (Lm+ group), 30 patients with negative SPT to grass pollen, but positive SPT to mite extracts (Lm– group), and 30 nonatopic subjects (NA group) were tested by SPT, ELISA, and immunoblot using Lm extract. Inhibition immunoassays with *Lolium perenne* (Lp), mixed grass (Gmix) and Lm extracts were also performed. **Results:** A high concordance was found between the Gmix and Lm extracts in SPT. Positivity rates in SPT were also highly concordant with IgE-ELISA results. The assay was able to detect Lm-specific IgE in >95% of Lm+ patients. A significant self- and cross-inhibition was observed in IgE-ELISA, reflecting a high cross-reactivity between the grass pollen allergens. Immunoblot revealed 13 IgE-binding Lm fractions, from which the bands 28–30 kDa and 31–34 kDa were recognized by >90% of Lm+ patients. **Conclusion:** Lm-specific IgE antibodies are highly cross-reactive with pollen proteins from other grass species. The results indicate that Lm extracts could be used in both SPT and ELISA for a more specific evaluation of IgE responses to Lm grass pollen in Brazilian pollinosis patients.

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Introduction

Lolium perenne (perennial ryegrass) pollen is a major source of grass allergen due to its broad distribution and abundant pollen production. Ninety percent of individuals allergic to ryegrass pollen exhibit IgE reactivities to Lol p 1 and Lol p 5 major allergens [1, 2].

Patients with grass pollen allergy, commonly also called pollinosis, often present reactivities to pollen allergens from a number of grass species due to cross-reactivity of IgE to pollen proteins present in both homologous and heterologous grasses [2]. In this context, Italian ryegrass (*Lolium multiflorum*) pollen of the Poaceae family cultivated in Southern Brazil has been considered a major sensitizing agent in patients with pollinosis [3]. The Italian ryegrass has been extensively used as an agriculturally viable livestock feed crop and as ground cover for soil protection, and is capable of producing around 100 kg of pollen per hectare. In addition to *L. multiflorum*, other Poaceae grasses such as *Paspalum notatum* (Bahia grass), *Anthoxanthum odoratum* (sweet vernal grass), *Cynodon dactylon* (Bermuda grass) and *Holcus lanatus* (velvet grass) are naturally grown as weed in Southern Brazil, but do not show any clinically relevant allergic responses [4].

An increased prevalence of pollinosis in Southern Brazil has been observed in the last decades. In an epidemiologic study performed between 1985 and 1986 in some cities in Southern Brazil, Vieira and Negreiros [3] found prevalence rates of grass pollen allergy ranging from < 1.0 to 4.8%. More recently, an ISAAC (International Study of Asthma and Allergies in Childhood) questionnaire with university students showed that the frequency of affirmative responses to seasonal rhinoconjunctivitis symptoms in the last 12 months was 14% in Caxias do Sul and 22% in Santo Angelo [5].

Pollen extracts derived from homologous or heterologous grasses are often used for both diagnosis and treatment of seasonal allergy. However, no standardized *L. multiflorum* extract is commercially available in Brazil and mixed grass extracts are commonly used for diagnosis and immunotherapy of grass pollen allergy. Furthermore, the grass species relevant to allergies have not been identified and whether *L. multiflorum* proteins are major allergens with IgE cross-reactivities to other grass pollen allergens remains to be determined.

We hypothesized that there is a high degree of IgE cross-reactivity among grass pollen allergens and aimed to investigate the sensitization of pollinosis patients to *L. multiflorum* in Southern Brazil.

Material and Methods

Pollen Sampling and Allergen Extraction

L. multiflorum grasses were naturally grown in farms of Caxias do Sul, RS, Brazil, and pollen grains were collected in October 2003, stored at 4°C in an airtight container, and processed at the Laboratory of Allergy and Clinical Immunology of Federal University of Uberlândia, MG, Brazil. Pollen grains were passed through a 56-µm mesh screen and the pollen purity based on optical microscopy was more than 99%. *L. multiflorum* (Lm) pollen extract was prepared as described elsewhere [6], with modifications. Briefly, 50 mg of pollen were stirred overnight at 4°C in 5 ml of phosphate-buffered saline solution (pH 7.2) in the presence of protease inhibitors (50 µg/ml leupeptin, 1.6 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 10 µg/ml aprotinin). Insoluble material was removed by centrifugation for 30 min at 20,000 g and the supernatant was extensively dialyzed against phosphate-buffered saline. Lm extract aliquots were stored at -20°C and the total protein content was determined by the method of Lowry et al. [7].

Patients

Sixty patients (29 male and 31 female; 32.4 ± 11.4 years) with a clinical history of rhinoconjunctivitis with or without mild to moderate asthma to grass pollen were selected among the patients attended in a private clinic by Dr. Francisco Vieira in Caxias do Sul, Southern Brazil, during the pollen season in October 2003. Among these, only 22 patients returned to the clinic out of pollen season in June 2004 and were reevaluated. As allergic patient control group, 13 subjects (3 male and 10 female, 23.9 ± 11.1 years) with rhinitis with/without mild to moderate asthma to house dust mites were recruited from Caxias do Sul. An additional control group was included by selecting 17 subjects (9 male and 8 female, 27.1 ± 6.7 years) with rhinitis/asthma to house dust mites in the Allergy Clinic at the Federal University of Uberlândia, located in a city in Midwestern Brazil, where pollen seasonal allergy is not commonly observed. Thirty healthy subjects (9 male and 21 female; 26.2 ± 9.3 years) from Uberlândia were also included as nonatopic control group. The exclusion criteria were: acute and chronic upper respiratory infections within 30 days before the study, use of antihistamines within the previous week, and topical or oral corticosteroids within the previous 4 weeks. The study was approved by the Ethics Committee in Human Research at the Federal University of Uberlândia and informed consent was obtained from all individuals.

Skin Prick Test and Serum Samples

Skin prick test (SPT) was performed using Lm pollen extract (0.4% phenol and 50% glycerin solution) at a final concentration of 1 mg/ml. The following commercially available glycerinated extracts were also used in SPT: pollen extracts of *L. perenne* (Lp) (Bayer Corp., Washington) and grass mix (Gmix) (IPI/ASAC, São Paulo, Brazil) consisting of the grass species *Dactylis glomerata*, *Festuca pratensis*, *Lolium perenne*, *Poa pratensis*, *Phleum pratense*, and mite extracts of *Dermatophagoides pteronyssinus* (Dp), *Dermatophagoides farinae* (Df) and *Blomia tropicalis* (Bt) (IPI/ASAC). As positive and negative controls, histamine (10 mg/ml) and 50% glycerin solution buffer were used, respectively. Skin test reactions were read after 15 min and positivity was defined by mean wheal size diameter of 3 mm or more than that of the negative control. Blood samples were collected and sera were stored at -20°C until serologic testing.

IgE-ELISA to L. multiflorum and Total Serum IgE

Specific IgE antibodies to *L. multiflorum* pollen were measured by ELISA as described [8], with modifications. Briefly, microtiter plates were coated with Lm extract (1 µg/well) and incubated with serum samples diluted 1:2. Biotinylated anti-human IgE (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Md., USA) was added followed by streptavidin-peroxidase (Sigma Chemical Co., St. Louis, Mo., USA). The ABTS and 0.03% H₂O₂ were used for color development and the reaction was read at 405 nm in an ELISA reader. Results were arbitrarily expressed as ELISA index (EI) and determined as follows: EI = absorbance of test sample/cutoff, where cutoff was calculated as the mean absorbance of negative control sera plus 5 standard deviations, according to our previous report [9]. EI values >1.2 were considered positive values, i.e., 20% above the corresponding positive value for excluding positive reactivity close to this threshold value. Total serum IgE was measured by a monoclonal antibody-based ELISA as previously described [8]. Results were expressed as international units (IU) per milliliter of serum calculated by comparison with a positive serum (LHY) containing 500 IU/ml of total serum IgE.

Lm-Specific IgE Inhibition Assays

The allergen specificity of the IgE-ELISA to Lm extract was determined using a competitive inhibition assay, according to Pereira et al. [10], with some modifications. Briefly, aliquots of 1:5 diluted pooled sera from 4 *L. multiflorum* allergic patients were incubated overnight with 10-fold diluted Lm, Lp, or Gmix dialyzed extracts to prevent inhibition of the assay by glycerol. Dp extract (unrelated allergen) and tetanus toxoid (TT, unrelated antigen) were included as controls. Residual uninhibited IgE reactivities to Lm were measured by ELISA and presented as percentage of activity at protein extracts with no inhibitors (inhibition %). For determining self- and cross-inhibition in individual sera, each sample was diluted to give an absorbance unit of 1.0 in standard Lm ELISA measurements (20- to 80-fold dilution) and incubated with 100 µg/ml of each extract. Residual IgE reactivities to Lm extract were assayed as described above.

SDS-PAGE and Immunoblot

Lm, Lp and Gmix (10 µg protein/lane) extracts were fractionated in 13.5% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels, and stained with Coomassie blue. After SDS-PAGE, unstained Lm protein gels were transferred to nitrocellulose membranes (Amersham, Pharmacia Biotech, Buckinghamshire, England) [11, 12], and IgE-reactive protein bands were visualized by chemiluminescence as described elsewhere [9], with some modifications. Briefly, strips containing about 10 µg of protein were blocked and incubated with serum samples diluted at 1:5. Biotinylated anti-human IgE diluted at 1:1,000 was incubated for 4 h at room temperature followed by streptavidin-peroxidase (1:1,000) for 2 h at room temperature. Bands were visualized by a chemiluminescence system (Amersham, Pharmacia Biotech).

Statistical Analysis

Differences between percentages of SPT positivity were analyzed by the chi-square test and the Fisher's exact probability test was used when one of the expected cells was less than 5. Differences in mean wheal size, total serum IgE and Lm-specific IgE levels among the groups were assessed by the Kruskal-Wallis test using as post-hoc test the Dunn's multiple comparison test. Comparison

between mean levels of IgE antibodies during and out of pollen season was performed by the Mann-Whitney test. Correlation between mean levels of IgE antibodies during and out of pollen season was determined by the Spearman test. Values of $p < 0.05$ were considered statistically significant.

Results

Patient Data

The demographic and immunological characteristics of the study subjects are shown in table 1. Subjects were divided into three groups according to their positivities in SPT to Lm and Gmix pollen extracts: Lm+ group (positive SPT to grass pollen extracts), Lm- group (negative SPT to grass pollen extracts, but positive SPT to at least one mite extract) including patients from Caxias do Sul (subgroup Lm-CS) and Uberlândia (subgroup Lm-Udia), and nonatopic (NA) group (negative SPT to pollen and mite extracts). All subjects showed a positive SPT to histamine. Rhinitis was the most frequently observed diagnosis and asthma much less frequently in the Lm+ and Lm- groups, but no significant difference in frequencies was found between the groups. In contrast, the frequency of conjunctivitis was higher in the Lm+ than in the Lm- group ($p < 0.0001$). In the Lm+ group, the mean wheal size in SPT was higher with Lm extract than Gmix extract, but the difference was not statistically significant. However, the same positivity rate (100%) in SPT was found for both extracts. SPT positivity to house dust mite extracts was higher in the Lm- than Lm+ group ($p < 0.0001$). The mean wheal size to all mite extracts was higher in the Lm-Udia subgroup than in the Lm+ group and Lm-CS subgroup, but a significant difference ($p < 0.001$) was noted only for Dp extract. Among patients of the Lm- group, SPT positivity rates found in subjects from Caxias do Sul and Uberlândia were, respectively, 100 and 82% for Dp extract, 54 and 88% for Df extract, and 70 and 82% for Bt extract. Overall, positive SPT rates to mite extracts were comparable between patients from Caxias do Sul (54–100%) and Uberlândia (82–88%) (data not shown). The mean total serum IgE levels in the Lm+ and Lm- groups were significantly higher ($p < 0.0001$) than in the NA group.

Specific IgE to Lm Pollen Allergen

All sera from Lm+ patients were positive for IgE-ELISA to Lm whereas Lm-specific IgE was detected only in 3.3% of both Lm- and NA groups (fig. 1). The mean levels of specific IgE in Lm+ patients were significantly

Fig. 1. Levels of specific IgE to Lm pollen in 60 serum samples from patients with positive SPT to Lm (Lm+ group), 30 sera from patients with negative SPT to Lm (Lm-group) and 30 sera from nonatopic subjects (NA group). Mean levels and positivity rates (%) are also shown. The dashed line indicates the positive threshold value for Lm-specific IgE (EI = 1.2). *** p < 0.0001.

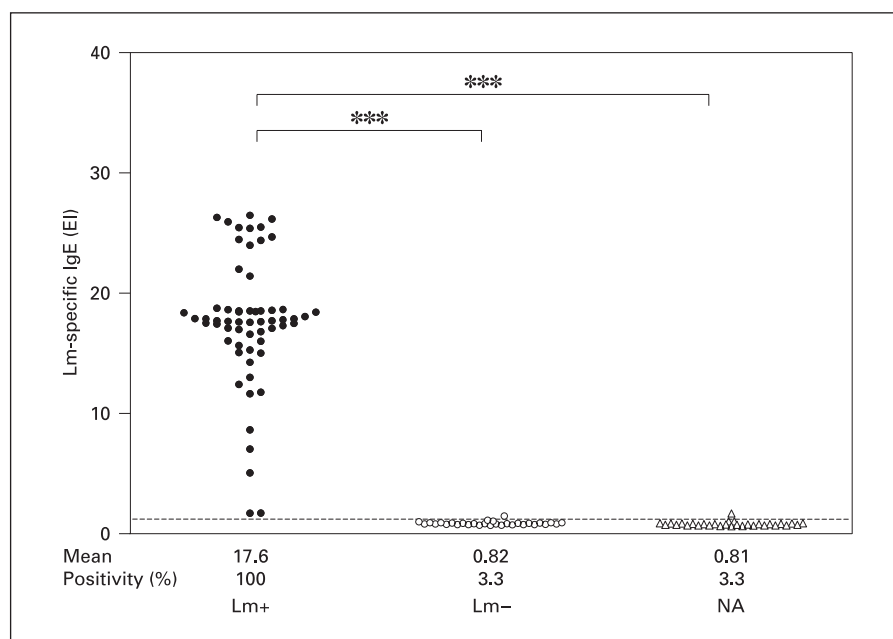


Table 1. Demographic and immunological characteristics of the study subjects

Characteristics	Groups			
	Lm+	Lm- Lm-CS	Lm-Udia	NA
Subjects, n	60	13	17	30
Mean age, years	32.4 ± 11.4	23.9 ± 11.1	27.1 ± 6.7	26.2 ± 9.3
Sex (M/F)	29/31	3/10	9/8	9/21
Clinical symptoms, n (%)				
Rhinitis	52 (86.7%) ^a	13 (100%) ^a	16 (94.1%) ^a	0
Conjunctivitis	47 (78.3%) ^a	7 (53.8%) ^a	0	0
Asthma	8 (13.3%) ^a	1 (7.7%) ^a	4 (23.5%) ^a	0
Positive SPT (mean wheal size, mm/positivity, %)				
<i>L. multiflorum</i>	11.1 ± 3.5 ^a 100% ^a	0 0	0 0	0 0
Grass mix	8.9 ± 2.5 ^a 100% ^a	0 0	0 0	0 0
<i>D. pteronyssinus</i>	5.2 ± 0.9 ^a 30% ^a	6.1 ± 1.3 ^a 100% ^b	10.2 ± 5.0 ^b 82% ^b	0 0
<i>D. farinae</i>	5.0 ± 0.7 ^a 15% ^a	6.1 ± 1.3 ^a 54% ^b	6.7 ± 2.8 ^a 88% ^c	0 0
<i>B. tropicalis</i>	5.4 ± 1.3 ^a 22% ^a	5.7 ± 1.3 ^a 70% ^b	7.2 ± 3.1 ^a 82% ^b	0 0
Mean total serum IgE, IU/ml	528.9 ± 1,101.0 ^a	581.3 ± 671.2 ^a	229.1 ± 346.5 ^b	10.14 ± 14.91 ^c

Lm+ = Patients with positive SPT to Lm; Lm- = patients with negative SPT to Lm; NA = nonatopic subjects.

^{a, b, c} Different letters indicate statistically significant differences between the subject groups, as indicated by the Kruskal-Wallis test using as post-hoc test the Dunn's multiple comparison test for mean wheal size and total serum IgE, and chi-square or Fisher's exact probability tests for SPT positivity (p < 0.05).

higher than those obtained in Lm- patients ($p < 0.0001$) and NA subjects ($p < 0.0001$).

Differences in IgE Antibody Levels during and out of Pollen Season

To investigate the individual variations of total serum IgE and Lm-specific IgE levels, 22 sera from Lm+ patients were collected during and out of pollen season (fig. 2). Mean total serum IgE levels during the pollen season were higher, but not significantly different from those in samples collected out of pollen season. In contrast, mean serum levels of Lm-specific IgE during the pollen season were significantly higher than those found out of pollen season ($p < 0.0001$). In addition, moderate and strong positive correlations were found in the levels of total ($r = 0.6228$; $p = 0.002$) and specific IgE ($r = 0.7944$; $p < 0.0001$), respectively, between the two pollen season periods analyzed (data not shown).

IgE-ELISA Self- and Cross-Inhibition with Related Grass Pollen Allergens

The anti-Lm IgE specificities were examined by competitive anti-Lm ELISA measurements using pooled sera from Lm+ patients. Lm pollen and related grass pollen extracts (Lp, Gmix) or unrelated allergen (Dp) and antigen (TT) were used as inhibitors. Lm extract inhibition (self-inhibition) resulted in a sigmoid dose-response curve as expected with maximal inhibition at 100 $\mu\text{g/ml}$ and half-maximal inhibition (I_{50}) at 2.5 $\mu\text{g/ml}$ of Lm extract (fig. 3a). Remarkably, Lp and Gmix inhibited anti-Lm IgE reactivities efficiently at maximal concentration (100 $\mu\text{g/ml}$) while at I_{50} only Lp showed similar inhibition to Lm extract, once I_{50} to Lp and Gmix were 3.4 and 18 $\mu\text{g/ml}$, respectively. Unrelated antigens (Dp and TT) exhibited negligible inhibition.

The pattern of grass pollen cross-reactivities was further examined in 10 representative sera from Lm-allergic patients using the maximal allergen inhibitory dose (fig. 3b). All three grass pollen extracts showed significant self- and cross-inhibition abilities. Lm self-inhibition was the highest in most sera analyzed ($84.2 \pm 13.1\%$) while the cross-inhibitions by Lp and Gmix extracts were 76.3 ± 11.9 and $69.3 \pm 13.1\%$, respectively. Significant difference was found only between Lm and Gmix inhibition ($p < 0.05$).

SDS-PAGE and Immunoblot of Grass Pollen Extracts

The three grass pollen extracts (Lm, Lp and Gmix) showed similar staining patterns in SDS-PAGE gels. The major protein bands varied in molecular weight from

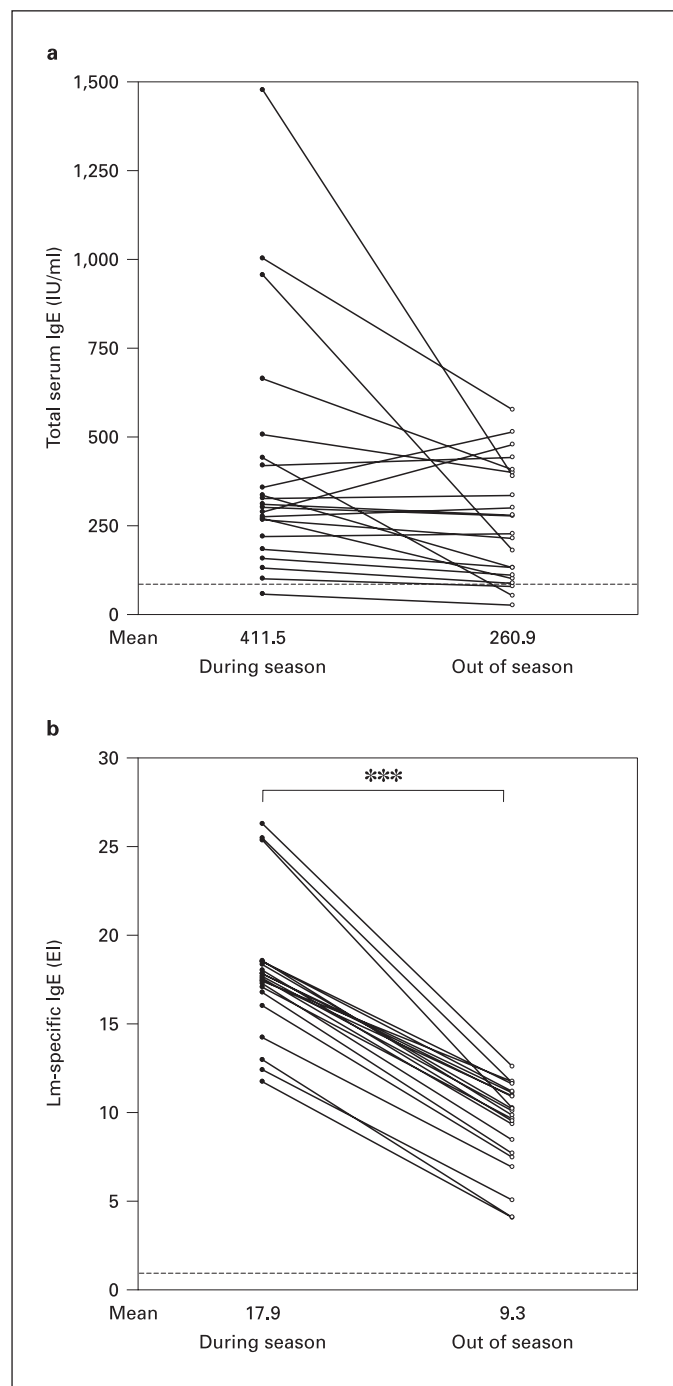


Fig. 2. Levels of total serum IgE (**a**) and Lm-specific IgE (**b**) during and out of pollen season in 22 sera from patients with positive SPT to Lm. The dashed lines indicate the positive threshold values for each reaction (total serum IgE = 100 IU/ml; Lm-specific IgE = 1.2 EI). Mean levels for each pollen season are also shown. *** $p < 0.0001$.

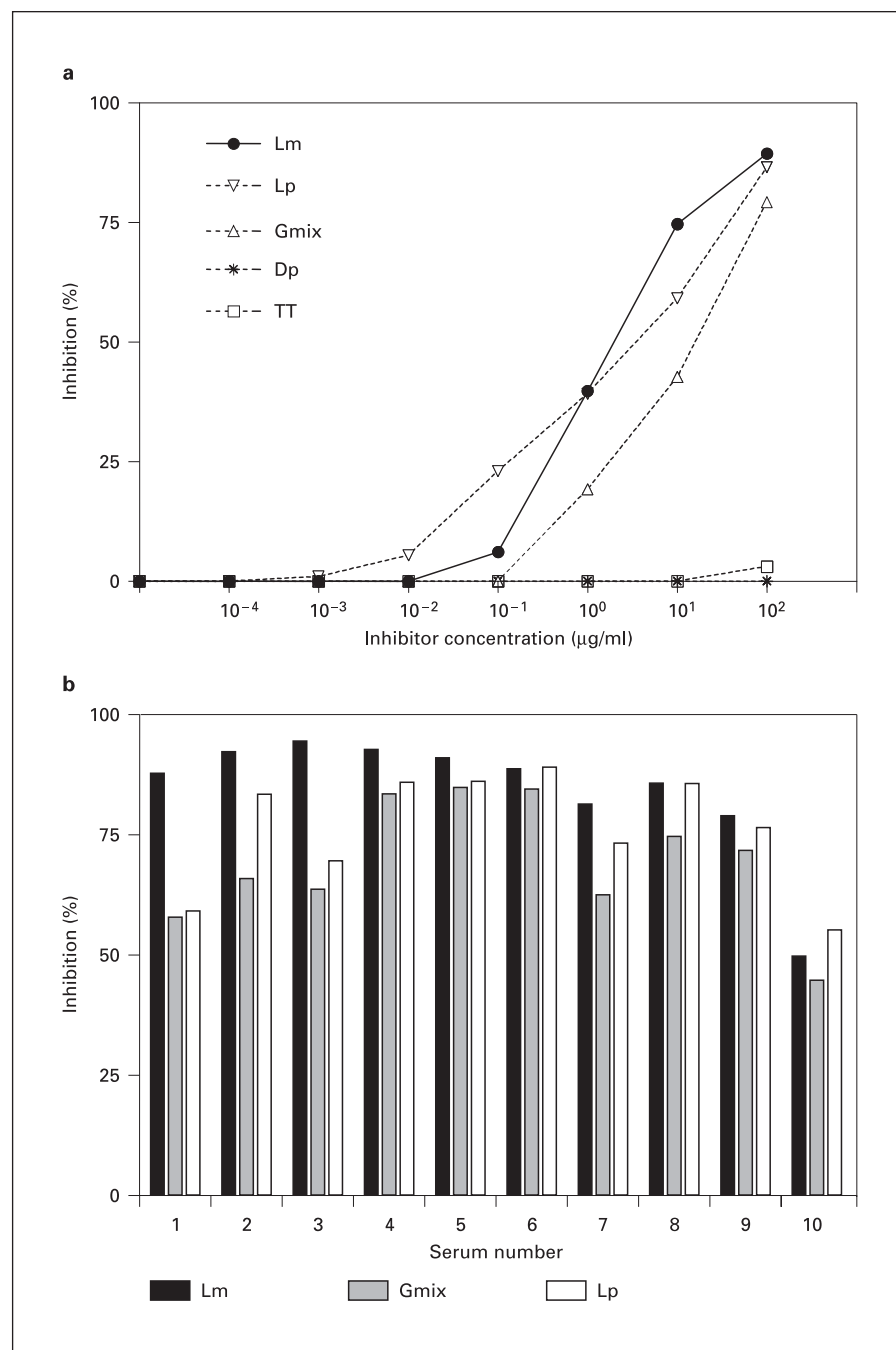


Fig. 3. a IgE-ELISA inhibition curves with Lm pollen extract on the solid phase. Pooled sera from Lm+ patients were preabsorbed overnight at 4°C with different concentrations of Lm, Lp, Gmix, Dp extracts and TT. **b** Results of cross- and self-inhibition analysis using 10 individual sera from Lm IgE-positive patients, which were preabsorbed as above with 100 μg/ml of each pollen extract.

about 15 to 114 kDa (15, 17, 19, 21, 26, 28–30, 31–34, 40, 45, 66, 75, 90, and 114 kDa) (fig. 4a).

One hundred and twenty sera from Lm+ (n = 60), Lm– (n = 30), and NA (n = 30) groups were used in IgE immunoblots to identify putative allergens in Lm extracts and representative sera from each group are illustrated in figure 4b. Sera showed similar IgE-binding pattern but with

varying degrees of differences in reactivities (fig. 4c). Thirteen Lm-allergenic components ranging from 15 to 114 kDa were recognized by IgE in sera from Lm+ patients. The allergenic fractions of 28–30 kDa showed the highest frequency of IgE recognition (96%) followed by the 31–34 kDa (93%), 55 kDa (64%), 17 and 21 kDa (62%), 19 and 45 kDa (60%) and 15 kDa (56%) major al-

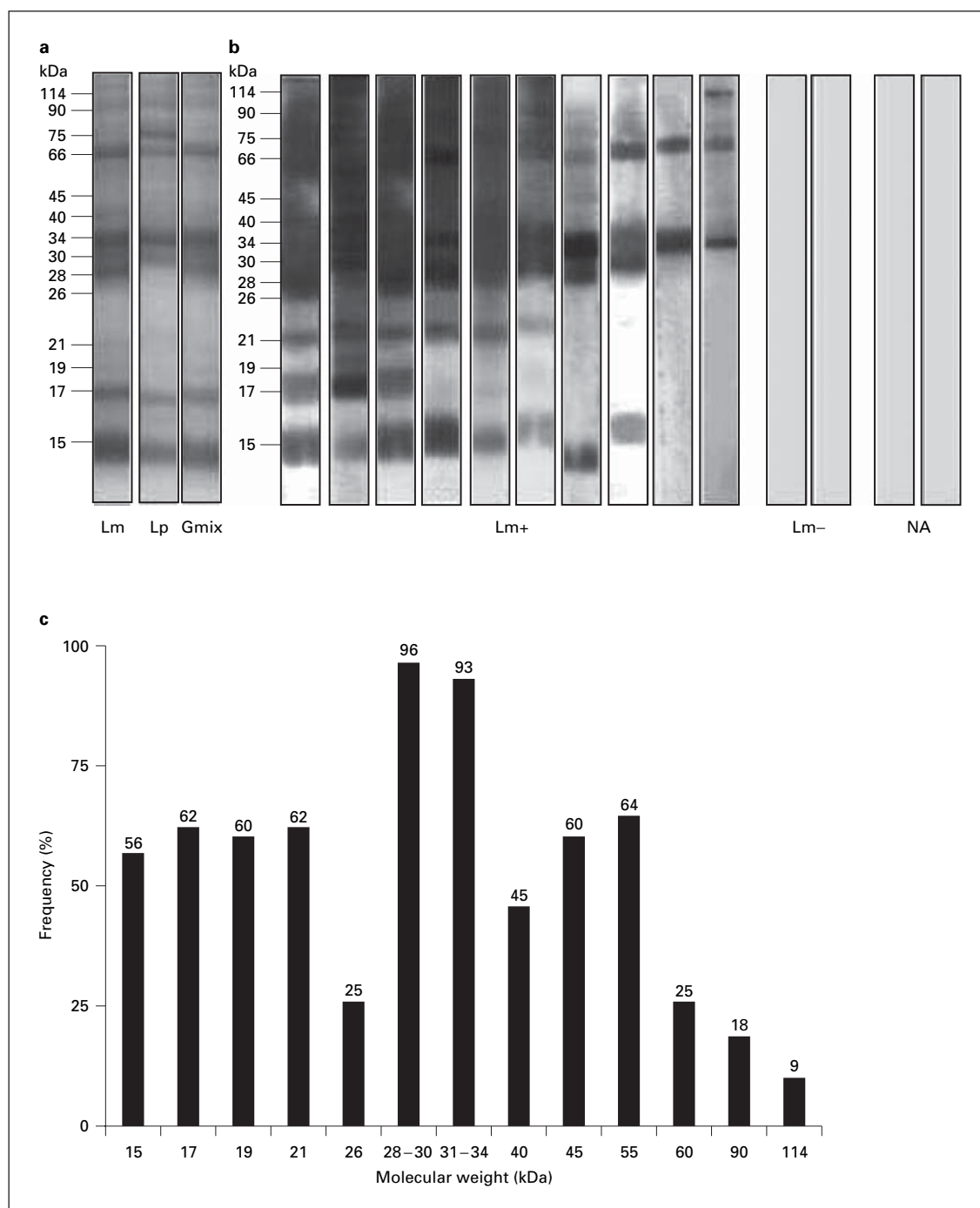


Fig. 4. SDS-PAGE profile of pollen extracts (**a**), IgE immunoblot of Lm pollen proteins (**b**), and frequency (%) of Lm protein bands recognized by IgE immunoblot in 60 sera from Lm IgE-positive patients (**c**). **a** Coomassie blue staining of Lm, Lp, and Gmix pollen extracts (10 μ g of protein). **b** Representative immunoblots from 10 patients' sera with positive SPT to Lm (Lm+ group), 2 from patients' sera with negative SPT to Lm (Lm- group), and 2 from nonatopic subjects' sera (NA group). Molecular weights (kDa) in **a** and **b** are indicated in the left margin.

lergenic fractions. Other minor allergenic fractions included the 40 kDa (45%), 26 and 60 kDa (25%), 90 kDa (18%) and 114 kDa (9%) bands (fig. 4c). In immunoblots of control sera, no allergenic component was noted (fig. 4b).

Discussion

Perennial ryegrass is the major allergen source causing grass pollen allergies in temperate zones [6, 13], although other grasses may be more prevalent in certain geographical regions [14, 15]. In Southern Brazil, Italian ryegrass (*L. multiflorum*) is a dominant allergenic source in the atmospheric environment. However, there have been no studies on the potencies of *L. multiflorum* in sensitization and the IgE antigenic specificities in *L. multiflorum*-allergic patients.

This study is the first to evaluate *L. multiflorum* pollen extract by using in vivo (SPT) and in vitro (ELISA and immunoblot) diagnostic tests. For comparison, a mixed grass pollen extract (Gmix), which is routinely employed in diagnosis and immunotherapy in Brazil, was used. However, this extract contains pollen allergens from mixed grasses, which are not cultivated in large scale in Southern Brazil. Among them, *L. perenne* grass pollen allergens could be clinically important in our country due to its largely related immunocross-reactivity with other grass pollen allergens [2]. On the other hand, there are several pollen allergens from existing Poaceae grasses in that region which are not included in the Gmix extract, such as *L. multiflorum* and *P. notatum* grasses.

We found a high concordance in SPT results between Gmix and Lm extracts. However, Lm extracts elicited slightly greater mean wheal size, suggesting a more specific IgE reactivity to Lm allergens in pollinosis patients. This higher IgE reactivity was further supported by the inhibition ELISA data showing that a lower I_{50} was obtained with Lm than Gmix extracts. Thus a homologous pollen extract, in this case Lm, is more appropriate for quantitative SPT tests. On the other hand, an alternative interpretation should be taken, since it is equally plausible that the extraction methods used in this study may have led to a more concentrated extract than that prepared by the commercial manufacturer.

Lm extract was also evaluated in vitro by an indirect ELISA for the quantitation of Lm-specific IgE. The high concordance between positivity rates in SPT and IgE-ELISA suggests that Lm-specific IgE-ELISA could be a valuable tool for evaluating IgE responses to Lm grass

pollen. In addition, the low positivity rates in Lm- and NA groups could be explained due to the high specificity of Lm-specific IgE-ELISA, since the Lm-positive IgE levels in Lm- and NA groups were extremely low (EI = 1.43 and 1.68, respectively) and closer to the cutoff value predetermined (EI = 1.2). It is worthy to note that in the Lm+ group only 2 patients showed similarly low, but positive Lm-specific IgE levels (EI = 1.67 and 1.68) while the 58 remaining patients presented high specific IgE levels (EI > 5.0). Probably, these 2 patients may not be primarily sensitized to Lm but to other grass pollen allergens that may be cross-reactive with Lm. Another possible explanation is that other grass (*C. dactylon*, *H. lanatus*, *A. odoratum* and *P. notatum*) and maize (*Zea mays*) pollens are present in the region where these subjects are living, thus reflecting a probable IgE cross-reactivity, but not enough to develop pollinosis symptoms in Lm- and NA subjects. It should also be noted that positive specific IgE cannot always be translated into clinical allergy, since immunochemical cross-reactivity between homologous epitopes expressed in different allergens has been identified as one of the main reasons for clinically irrelevant IgE [16]. Clinically relevant cross-reactivity between different grass pollen allergens is well known and has also been shown to be due to homologous epitopes. Most cross-reactive grass pollen allergens exhibit a high degree of homology at the primary amino acid sequence level, which is also a reflection of their phylogenetic relationship. The most likely explanation for the lack or poor clinical relevance of some of these cross-reactive IgE antibodies may be due to low total allergen epitope valences or low affinity [17].

Mite allergens are important sensitizing agents in allergic diseases, particularly asthma and rhinitis. In temperate climates, *D. pteronyssinus* and *D. farinae* are predominant mites, but *B. tropicalis* may also be an important sensitization source in tropical countries [10]. In our study, positivity rates in SPT to mites were lower in the Lm+ group than in the Lm- group, indicating that the majority of the grass pollen-sensitized patients were not concomitantly sensitized with mite allergens and the predominant causative allergen of rhinoconjunctivitis in these patients is probably related to pollen sensitization. On the other hand, the high mite SPT positivity in patients of the Lm- group reflects the importance of the mite allergens in the sensitization of these patients in presenting rhinitis as prevailing diagnosis rather than conjunctivitis. In addition, no difference in overall SPT positivity to mites between Lm- patients living in areas with (Caxias do Sul) and without (Uberlândia) pollinosis was found.

Therefore, we hypothesize that sensitization to mite allergens could be independent of grass sensitization once mite skin reactivity was similar in these two geographic regions.

Total serum IgE is known to be increased in mite- and pollen-sensitized patients [18, 19]. In this context, atopic patients in both Lm+ and Lm- groups showed remarkably higher mean levels of total serum IgE than nonatopic subjects, reflecting the importance of this general parameter in both diagnosis and management of allergic patients. Exposure to grass pollen allergens immediately triggers symptoms in pollen-sensitized patients. Thus, specific IgE levels decline toward a basal level before, and increase after, the grass-pollination season [19]. In this study, total serum IgE and Lm-specific IgE were higher during than out of pollen season in the Lm+ group, a similar finding to that of Beeh et al. [20]. However, Lm-specific IgE levels still maintained positive out of the pollen season, suggesting that diagnosis made in both periods are legitimate.

For the first time, this study investigated the IgE reactivities and specificities in pollinosis patients to Lm pollen allergens. In the evaluation of IgE specificities using *L. multiflorum* extract proteins as the immobilized antigen, strong self- and cross-inhibition were observed. The results reflect a high degree of cross-reactivity among the pollen allergens present in Lm and Lp extracts. However, it is difficult to state, based on the inhibition ELISA, that Lm shows cross-reactivity with other grasses present in the Gmix since the Lp allergens in Gmix extract may have been responsible for the inhibition of IgE binding to Lm extract. In the present study, a very similar electrophoretic profile was visualized among the Lm, Lp and Gmix extracts. Although the identities of the proteins were not confirmed, the major protein bands at 28–30 kDa and 31–34 kDa likely correspond to the group 5 and group 1 pollen allergens, respectively, and were recognized by sera from Lm+ patients most often (>90%). These results are in agreement with the findings of Singh and Knox [21] and Sch  ppi et al. [22] that detected group 1 and group 5 pollen allergens using specific monoclonal antibodies. Among these, the monoclonal 1D11 is a Phl p 5-specific antibody that recognizes the band of 30 kDa in many grasses, including *L. perenne* and *L. multiflorum*.

In addition, six other pollen allergenic components ranging from 23 to 80 kDa in ryegrass pollen have been suggested to be clinically important in at least a large proportion (33–50%) of the pollen-sensitized patients [1]. The 15-, 17-, 19-, 21-, 45-, and 55-kDa protein bands were recognized by IgE in the sera of >50% of the patients in

the Lm+ group. The 15-kDa protein could be related to the 10- to 12-kDa group 2 or group 3, or the 16-kDa group 11 allergens [7, 23]. The 55-kDa protein may be correspondent to the group 4 pollen allergens which have been characterized as a 59-kDa protein in *L. perenne* and a 55-kDa protein in *P. pratense* [24, 25]. Alternatively, this 55-kDa protein may be related to the group 13 protein Phl p 13 [25, 26]. Further characterization of these Lm proteins by IgE immunoblot inhibition with other grass species, inhibition or direct binding studies with specific antibodies, isoelectric focusing, peptide mapping, peptide sequencing, or cDNA cloning will confirm the relationship of these proteins with established allergens.

In this study, two high molecular weight IgE-binding Lm proteins (90 and 114 kDa) were poorly recognized (<20%) by sera from Lm+ patients. In contrast, Ford and Baldo [23] found that the 100-kDa protein in *C. dactylon* was recognized by the majority of the allergic patients. The low IgE reactivity frequencies found for proteins in the 100-kDa molecular weight range (90 kDa, 18% and 114 kDa, 9%) in Lm+ patients may be explained by the absence of these reactivities in sera of pollen-sensitized patients against *L. perenne* extracts [1, 21].

Our results showed that patients with pollinosis in Southern Brazil exhibit strong IgE reactivities against allergenic components present in the Lm extract, which showed a great similarity and high cross-reactivities to proteins present in Lp and Gmix extracts.

This study confirms the complexity of grass pollen extracts and the importance of identifying IgE-binding protein patterns in novel and clinically important allergens from Poaceae grass pollen. Further studies are required to better characterize the cross-reactivity between Lm and other grass pollen allergens for improving the diagnosis and immunotherapy to *L. multiflorum* pollen allergy.

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