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IgE cross-reactivity between *Lolium multiflorum* and commercial grass pollen allergen extracts in Brazilian patients with pollinosis

C.T. Bernardes[1], P.F. Moreira[1], M.C. Sopelete[1], F.A. Vieira[2], S.S. Sung[1], D.A. Silva[1] and E.A. Taketomi[1]

[1]Laboratório de Alergia e Imunologia Clínica, Instituto de Ciências Biomédicas, Universidade Federal de Uberlândia, Uberlândia, MG, Brasil
[2]Departamento de Medicina, Universidade de Caxias do Sul, Caxias do Sul, RS, Brasil

Abstract

Lolium multiflorum (Lm) grass pollen is the major cause of pollinosis in Southern Brazil. The objectives of this study were to investigate immunodominant components of Lm pollen allergens and the cross-reactivity of IgE with commercial grass pollen allergen extracts. Thirty-eight serum samples from patients with seasonal allergic rhinitis (SAR), 35 serum samples from patients with perennial allergic rhinitis (PAR) and 30 serum samples from non-atopic subjects were analyzed. Allergen sensitization was evaluated using skin prick test and serum IgE levels against Lm pollen extract were determined by ELISA. Inhibition ELISA and immunoblot were used to evaluate the cross-reactivity of IgE between allergens from Lm and commercial grass pollen extracts, including *L. perenne* (Lp), grass mix I (GI) and II (GII) extracts. IgE antibodies against Lm were detected in 100% of SAR patients and 8.6% of PAR patients. Inhibition ELISA demonstrated IgE cross-reactivity between homologous (Lm) and heterologous (Lp or GII) grass pollen extracts, but not for the GI extract. Fifteen IgE-binding Lm components were detected and immunoblot bands of 26, 28-30, and 32-35 kDa showed >90% recognition. Lm, Lp and GII extracts significantly inhibited IgE binding to the most immunodominant Lm components, particularly the 55 kDa band. The 26 kDa and 90-114 kDa bands presented the lowest amount of heterologous inhibition. We demonstrated that Lm extract contains both Lm-specific and cross-reactive IgE-binding components and therefore it is suitable for measuring quantitative IgE levels for diagnostic and therapeutic purposes in patients with pollinosis sensitized to Lm grass pollen rather than other phylogenetically related grass pollen extracts.

Key words: Pollinosis; Allergic rhinoconjunctivitis; Grass pollen allergen; *Lolium multiflorum*; Cross-reactivity

Introduction

Grass pollen allergy is a common cause of morbidity in genetically predisposed subjects. Furthermore, an increasing number of grass pollen species have been recognized as important allergen sources (1-3). Among the different types of allergenic grass pollen species, Poaceae family plants such as *Lolium perenne*, *Poa pratensis* and *Phleum pratense* are the major sources of grass pollen allergens due to their broad distribution in certain geographic areas and high capacity of pollen production (4,5). *Lolium multiflorum* (Italian ryegrass) is the most important Poaceae grass related to pollinosis in the South region of Brazil (6), although other grasses of the same family, such as *Paspalum notatum* (Bahia grass), *Anthoxanthum odoratum* (sweet vernal grass), *Cynodon dactylon* (Bermuda grass), and *Holcus lanatus* (velvet grass), grow naturally as weeds in Southern Brazil, but they are not clinically relevant (7,8).

According to the International Union of Immunological Societies - Allergen Nomenclature Subcommittee (IUIS) (9), there are 13 major grass allergen groups based on molecular weight, structural homology and cross-reactivity among different grass species. Groups 1 and 5 are the most clinically relevant ones, since they are recognized by IgE antibodies in 95% of patients with pollinosis (10). Group 5 exclusively involves the Pooideae subfamily, whereas group

Correspondence: E.A. Taketomi, Laboratório de Alergia e Imunologia Clínica, Instituto de Ciências Biomédicas, UFU, Av. Pará, 1720, Campus Umuarama, Bloco 4C, 38400-902 Uberlândia, MG, Brasil. Fax: +55-34-3232-8620. E-mail: eat4y@yahoo.com.br

The present address of S.S. Sung is Department of Medicine, Center for Immunity, Inflammation and Regenerative Medicine, University of Virginia, Charlottesville, VA, USA.

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1 has also been identified in members from other subfamilies (11). Groups 2, 3, 4, and 13 show reactivity to serum IgE from about 50% of patients with pollinosis (12-14).

Immunochemical studies have shown similarities among grass pollen allergen-conserved epitopes (10,13), and the major allergens from groups 1, 2, 3, and 5 have demonstrated extensive cross-reactivity among Poaceae grasses (10). However, it is important to emphasize that there are specific allergens in some grass species such as *P. notatum* grass pollen that demonstrate little cross-reactivity with *L. perenne* and other relevant Poaceae grass species (5).

There is limited information on the major *L. multiflorum* allergenic fractions recognized by serum IgE antibodies in patients with pollinosis, especially those involving allergenic cross-reactivity, as already described in our previous study between *L. multiflorum* and other grass pollen allergen extracts (15). Thus, the objectives of the present study were to investigate the immunodominant components of an *L. multiflorum* pollen extract and to assess their cross-reactivity with grass pollen allergen extracts commonly used for the diagnosis and specific immunotherapy of pollinosis in Southern Brazil.

Patients and Methods

L. multiflorum pollen extract

L. multiflorum pollen grains were collected from grasses naturally growing in farms of Caxias do Sul, RS, Brazil. Pollen was purified by sieving and stored at 4°C for further extraction. *L. multiflorum* pollen extracts (Lm) were prepared using phosphate-buffered saline, pH 7.2, as previously described (15), stored at -20°C and the total protein content was determined by the method of Lowry et al. (16).

Patients and non-atopic subjects

Thirty-eight patients (14 males and 24 females, mean age: 32 ± 10.5 years) with a clinical history of seasonal allergic rhinoconjunctivitis (SAR) in response to grass pollen associated or not with mild-to-moderate asthma were selected at a private clinic in Caxias do Sul, a city in Southern Brazil, during the pollen season in October 2005 (SAR group). Nineteen patients (6 males and 13 females, mean age: 23.7 ± 10.9 years) with perennial allergic rhinitis (PAR) in response to house dust mites were also recruited from Caxias do Sul (PAR_{CS}) and used as controls. Another control group consisting of 16 patients (8 males and 8 females, mean age: 27 ± 7 years) with PAR in response to house dust mites was selected from the Allergy Unit at the Federal University of Uberlândia, a city in Midwestern Brazil, a region where pollinosis is not usually observed (PAR_{UDI}). Thirty healthy subjects (11 males and 19 females, mean age: 27 ± 10.5 years) from Uberlândia were also selected as a non-atopic (NA) control group. As additional inclusion criteria, SAR patients should have a positive skin prick test (SPT) to the Lm extract, PAR patients should have a positive SPT to mite extracts, but a negative SPT to the Lm extract, and NA patients should have a negative SPT to any allergenic extracts tested. The exclusion criteria were acute or chronic upper respiratory infections within 30 days before the study, use of anti-histamines 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 written consent was obtained from all participants.

Skin prick test

Allergenic sensitization to *L. multiflorum* pollen allergens was assessed by SPT using the Lm extract at a final concentration of 1 mg/mL in 0.4% phenol and 50% glycerin saline. The following commercially available glycerinated extracts were also used: grass mix II (GII: *L. perenne*, *Dactylis glomerata*, *Festuca pratensis*, *Poa pratensis*, and *Phleum pratense*) and mite extracts of *Dermatophagoides pteronyssinus* (Dp), *D. farinae* (Df) and *Blomia tropicalis* (Bt). Histamine (10 mg/mL) and 50% glycerin saline were used as positive and negative controls, respectively. All allergen extracts and control solutions were purchased from IPI-ASAC Brasil, Brazil. Reactions were read after 15 min and the result was considered to be positive when the mean wheal size diameter was 3 mm larger than the negative control. Blood was collected from all participants and sera were stored in aliquots at -20°C until serological assay.

ELISA for the detection of serum IgE antibodies to *L. multiflorum* pollen allergens

Serum IgE antibodies to *L. multiflorum* pollen allergens were measured by ELISA (15) using a biotinylated anti-human IgE (Kirkegaard and Perry Laboratories Inc., USA). Results are reported as ELISA index (EI) as previously proposed (15) and calculated as follows: EI = absorbance of test sample/cut-off, where cut-off was established as the mean absorbance of negative control sera plus five standard deviations. EI values >1.2 were considered to be positive.

Inhibition ELISA for IgE cross-reactivity

Inhibition ELISA was carried out to determine IgE cross-reactivity between Lm extract and commercial grass pollen allergen extracts (15,17,18). Briefly, the Lm extract was applied to the solid phase (1 µg/well) and six pooled sera (diluted 1:5) from SAR patients with high and similar levels of specific IgE antibodies to Lm pollen allergens (EI values ≅10) were pre-adsorbed overnight at 4°C with the following previously dialyzed inhibitor extracts: Lm, *L. perenne* (Lp), grass mix I (GI: commercial extracts containing a mix of the grass pollen allergens: *Avena sativa*, *Hordeum vulgare*, *Secale cereale*, and *Triticum sativum*) and GII. Also, the Bt extract was used as an unrelated inhibitor and all inhibitor extracts were tested in double serial decimal dilutions, with

concentrations of total protein ranging from 2 x 10^2 to 2 x 10^{-5} µg/mL. Sera with no inhibitors were used as positive control (100% reactivity). Residual uninhibited IgE levels in response to the Lm extract were measured by ELISA as mentioned above, and are reported as percent inhibition compared to the positive control.

SDS-PAGE and immunoblot

Lm extract proteins were separated by 13.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions (19) and transferred to nitrocellulose membranes (Hybond-C, Amersham, UK) (20). IgE-reactive protein bands were visualized by immunoblot, with some modifications (15). Briefly, strips containing about 20 µg protein were blocked and incubated with serum samples diluted 1:5. Biotinylated anti-human IgE diluted at 1:250 was incubated for 4 h at room temperature followed by the addition of the streptavidin-biotinylated peroxidase complex (ABC/HRP, Dako Corporation, USA) diluted 1:250 for 2 h at room temperature. Protein bands were visualized by adding 0.03% H_2O_2 and 3,3'-diaminobenzidine (Sigma, USA) and the molecular weights were estimated by calculating the relative mobility and comparison with standard proteins using the Kodak Digital Science 1-D image software (Eastman Kodak, USA).

Inhibition immunoblot

Inhibition immunoblot was used to determine the cross-reactive allergens between Lm extract and commercial grass pollen extracts. Ten sera from SAR patients (diluted 1:5), showing IgE reactivity to all immunodominant Lm bands (allergenic fractions recognized by >50% of patients) in a conventional immunoblot were pre-adsorbed with Lm, Lp, GI, and GII inhibitor extracts at maximal final concentration (200 µg/mL) for 2 h at 37°C and then centrifuged at 10,000 *g* for 5 min. The Bt extract was used as an unrelated inhibitor and sera with no inhibitors were used as positive control. After SDS-PAGE and electrotransfer of the Lm extract to nitrocellulose membranes, strips were blocked and incubated with the supernatants of pre-adsorbed serum samples for 18 h at room temperature. Subsequent steps were performed as described above for conventional immunoblots. Band intensity was determined in pixels using the Kodak Digital Science 1-D software (Kodak). Data are reported as percent inhibition compared to the positive control with no inhibitors.

Statistical analysis

All data were analyzed using the GraphPad Prism software version 4.0 (GraphPad Software Inc., USA). The Fisher exact probability test was applied to analyze the differences between percentages of positive SPT between groups and frequency of clinical features. As the data followed a non-Gaussian distribution, non-parametric testes were used. The Kruskal-Wallis test and the Dunn multiple comparison *post hoc* test were used to compare Lm-specific IgE levels and wheal size between groups. P values <0.05 were considered to be statistically significant.

Results

Patient clinical features and skin prick test

The clinical features and SPT results for patients of the atopic groups (SAR, PAR_{CS} and PAR_{UDI}) are shown in Figure 1. Rhinitis was the most frequent clinical manifestation observed in all atopic patients (94 to 100%), with no significant difference between groups ($P > 0.05$). In contrast, conjunctivitis was predominantly observed in SAR patients (92%) compared to the other two atopic groups (PAR_{CS} and PAR_{UDI}), with only PAR_{CS} patients having a history of conjunctivitis (42%; $P < 0.05$). Asthma was the least frequent clinical symptom (2.6 to 13%), with no significant difference between patient groups (Figure 1A).

SPT results showed a larger wheal size in response to the Lm extract (mean ± SD: 10.3 ± 2.3 mm) than the GII (5.0 ± 2.0 mm) extract ($P < 0.05$) in SAR patients (Figure 1B), even though presenting the same positivity rate (100%; Figure 1C). Positive SPT percentages in response to mite extracts were lower in SAR (16 to 32%) than PAR_{CS} and PAR_{UDI} (90 to 100%) patients ($P < 0.05$; Figure 1C), although the wheal sizes were significantly smaller only for the Dp and Df allergen extracts (Figure 1B). In addition, the mean wheal size and positive SPT rates using mite extracts were similar for the PAR_{UDI} and PAR_{CS} subgroups ($P > 0.05$; Figure 1B,C).

ELISA for IgE to *L. multiflorum* and inhibition ELISA

All sera from SAR patients (100%) were positive for IgE in response to the Lm extract, whereas only 3 (8.6%) sera from PAR patients showed Lm-specific IgE positivity, and NA subjects showed no IgE reactivity in ELISA (Figure 2A). Levels of Lm-specific IgE were significantly higher in SAR (median EI: 12.04) compared to PAR (median EI: 0.82) and NA (median EI: 0.76) patients ($P < 0.0001$), with no significant difference between the two latter groups ($P > 0.05$; Figure 2A).

The degree of IgE cross-reactivity between Lm extract and commercial grass pollen extracts was determined by inhibition ELISA using pooled sera from SAR patients pre-adsorbed with inhibitor extracts (Figure 2B). A high homologous inhibition by the Lm extract was observed, as demonstrated by sigmoid dose-response curves, reaching 87% inhibition at the maximal inhibitory concentration of 200 µg/mL, and the median inhibitory concentration (IC_{50}) was 12.3 µg/mL. Heterologous inhibition was also noted with the Lp and GII extracts, showing 52% inhibition of IgE in response to Lm at maximal inhibitory concentration, and IC_{50} was 190.5 µg/mL (for the Lp extract) and 197.2 µg/mL (for the GII extract). The GI and Bt extracts exhibited negligible inhibition (0 to 8%) of IgE reactivity to the Lm extract.

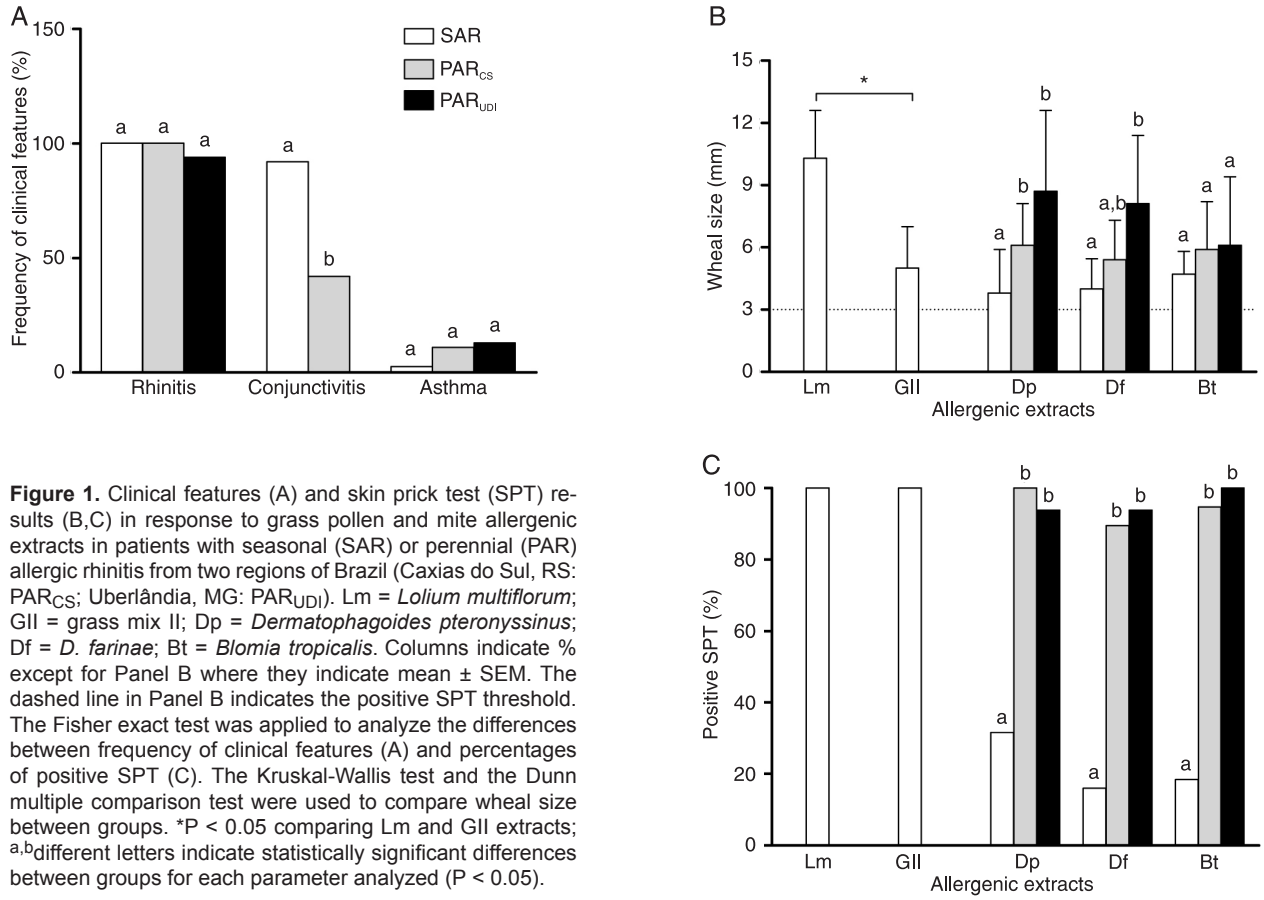

Figure 1. Clinical features (A) and skin prick test (SPT) results (B,C) in response to grass pollen and mite allergenic extracts in patients with seasonal (SAR) or perennial (PAR) allergic rhinitis from two regions of Brazil (Caxias do Sul, RS: PAR_{CS}; Uberlândia, MG: PAR_{UDI}). Lm = *Lolium multiflorum*; GII = grass mix II; Dp = *Dermatophagoides pteronyssinus*; Df = *D. farinae*; Bt = *Blomia tropicalis*. Columns indicate % except for Panel B where they indicate mean ± SEM. The dashed line in Panel B indicates the positive SPT threshold. The Fisher exact test was applied to analyze the differences between frequency of clinical features (A) and percentages of positive SPT (C). The Kruskal-Wallis test and the Dunn multiple comparison test were used to compare wheal size between groups. *P < 0.05 comparing Lm and GII extracts; [a,b]different letters indicate statistically significant differences between groups for each parameter analyzed (P < 0.05).

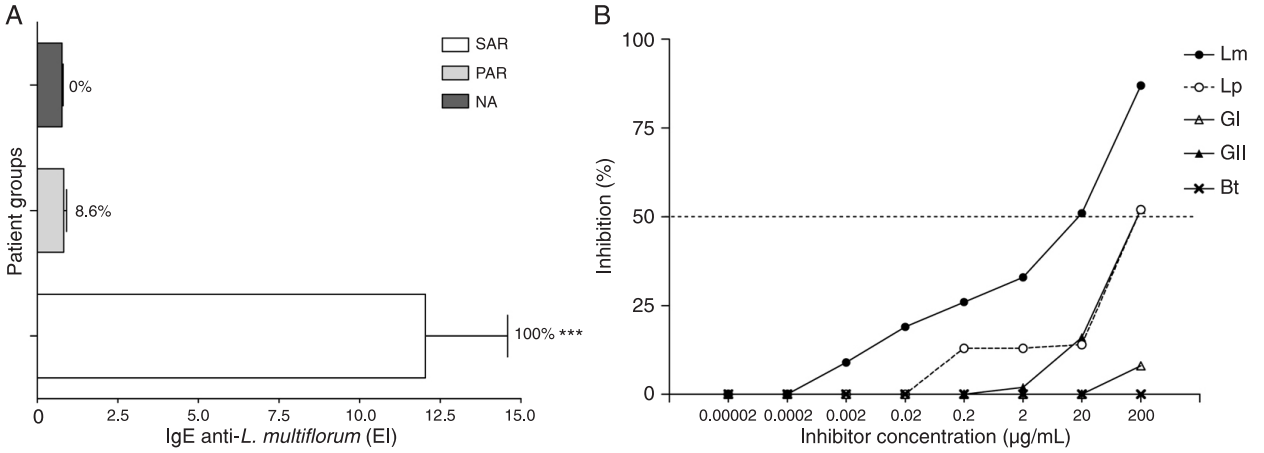

Figure 2. *A*, IgE levels in response to *Lolium multiflorum* grass pollen extract (Lm), reported as ELISA index (EI) in serum samples from patients with seasonal allergic rhinitis (SAR, N = 38) or perennial allergic rhinitis (PAR, N = 35), and from non-atopic subjects (NA, N = 30). Columns indicate the median and interquartile range. EI values >1.2 were considered to be positive. Percentages of positive samples for each group are also reported. ***P < 0.001 compared to the PAR and NA groups. B, Inhibition IgE-ELISA curves using Lm extract adsorbed to the solid phase and the following inhibitor extracts: *L. multiflorum* (Lm), *L. perenne* (Lp), grass mix I (GI), grass mix II (GII), and *Blomia tropicalis* (Bt) - submitted to serial decimal dilutions from 2 x 10^2 to 2 x 10^{-5} µg/mL and pre-incubated with pooled sera from 6 SAR patients. Percent inhibition was calculated by the residual antibody reactivity in relation to 100% reactivity (sera with no inhibitors). The dashed horizontal line intercepts the inhibition curves at the median inhibitory concentration (IC_{50}).

Immunoblot for IgE in response to *L. multiflorum* and inhibition of the immunoblot

A total of 103 serum samples from the SAR (N = 38), PAR (n = 35) and NA (N = 30) groups were tested in IgE immunoblots to identify allergenic components in the Lm extract and representative immunoblots of each group are illustrated in Figure 3A. A similar IgE-binding profile was observed with sera from SAR patients, but with reactivity variations depending on the antibody levels found in each serum sample. Fifteen allergenic components of the Lm extract ranging in molecular mass from 14 to 114 kDa (14, 19, 21, 23, 24, 26, 28-30, 32-35, 40, 55, 60-66, 72, 78, 84, and 90-114 kDa) were recognized by IgE in sera from SAR patients (Figure 3B). A total of eight allergenic fractions recognized by >50% of patients were considered to be immunodominant, such as the 26-kDa (95%), 28-30- and 32-35-kDa (92%), 90-114-kDa (84%), 60-66-kDa (76%), 72-kDa (68%), 40-kDa (63%), and 55-kDa (55%) bands. Other minor allergenic fractions having reactivity to IgE included the 14-kDa (42%), 19- and 21-kDa (37%), 78- and 84-kDa (37%), 23- and 24-kDa (34%) bands. Sera from the PAR and NA groups showed no IgE reactivity to the Lm extract.

IgE cross-reactive components between Lm extract and commercial grass pollen (Lp, GI, and GII) extracts were identified by inhibition immunoblot, and representative immunoblots of three sera from SAR patients with the different inhibitors are shown in Figure 4. There was a high percentage of homologous inhibition (82 to 100%) for all allergenic fractions (Figure 5A-C). Considering the Lp extract as an inhibitor (Figure 5A), two immunodominant bands (26 and 90-114 kDa) showed less than 50% inhibition, whereas only one (90-114 kDa) of them showed <50% inhibition by the GII extract (Figure 5C). No immunodominant bands presented >50% inhibition when the GI extract was used (Figure 5B). The non-immunodominant bands of 14, 19, and 21 kDa were the most cross-reactive components since they were totally inhibited by all grass pollen extracts tested (Figure 5A-C).

In the analysis of a more restricted range of Lm immunodominant bands (26 to 72 kDa), only the band of 26 kDa showed <50% inhibition when the Lp extract was used as inhibitor (Figure 5A), while all of these fractions were considerably inhibited (>50%) when the GII extract was tested (Figure 5C). Considering a degree of 80% as a high inhibition cut-off, only the Lm extract showed inhibition of all immunodominant allergenic components, whereas the GII extract showed >80% inhibition only for the im-

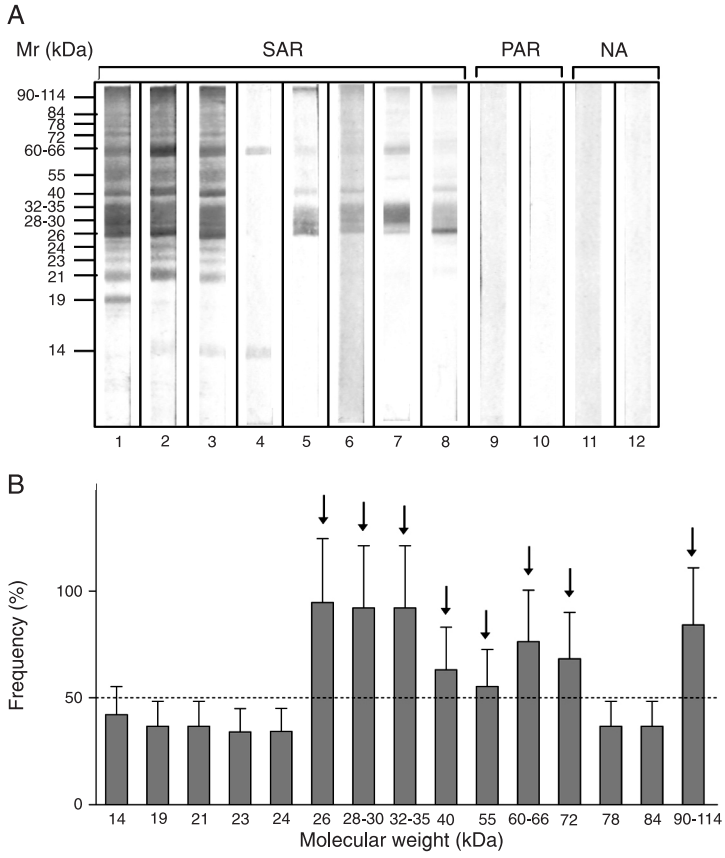

Figure 3. *A*, Representative immunoblots of IgE-binding components from *Lolium multiflorum* pollen extract (Lm). *Lanes 1* to *8* contain serum samples from patients with seasonal allergic rhinitis (SAR group); *lanes 9-10* are serum samples from patients with perennial allergic rhinitis (PAR group) and *lanes 11-12* contain serum samples from non-atopic subjects (NA group). Relative molecular masses (Mr) are indicated on the left in kDa. *B*, Frequency (%) of allergenic bands in the Lm extract recognized by IgE antibodies in serum samples from SAR patients (N = 38) by immunoblot. Columns represent the mean and 95% confidence intervals. The dashed horizontal line indicates 50% recognition. The arrows indicate the most frequent bands (>50% recognition).

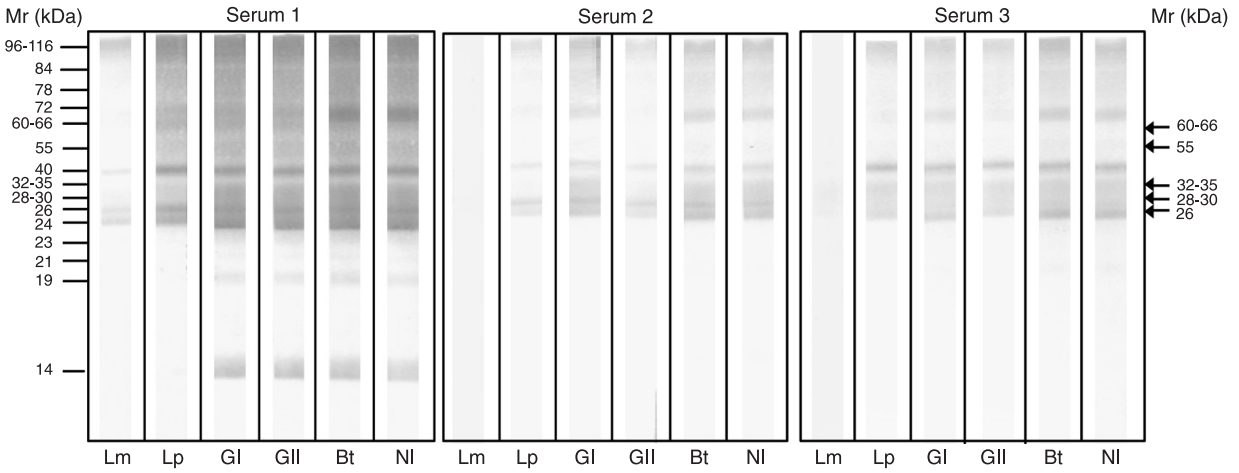

Figure 4. Representative inhibition immunoblots performed to identify IgE cross-reactive components between *Lolium multiflorum* pollen extract (Lm) and commercial grass pollen extracts in three serum samples from patients with seasonal allergic rhinitis (SAR group). The letters under each lane indicate the inhibitor extract used in pre-adsorption with serum samples. *Lm lane* = *L. multiflorum*; *Lp lane* = *L. perenne*; *GI lane* = grass mix I; *GII lane* = grass mix II; *Bt lane* = *Blomia tropicalis*; *NI lane* = no inhibitor. Relative molecular masses (Mr) are indicated on the left and right sides of the figure in kDa. The arrows on the right indicate the major components recognized by IgE (>90% recognition).

munodominant band of 55 kDa. Lm components exhibited negligible inhibition of IgE reactivity when the unrelated Bt mite allergen extract was used as inhibitor.

Discussion

Italian ryegrass (*L. multiflorum*) is the major allergen source for rhinoconjunctivitis in Southern Brazil. In a previous study, we evaluated *in vivo* and *in vitro* diagnostic tests of the Lm extract and suggested that the Lm extract could be used for a more specific evaluation of the IgE response to grass pollen in Brazilian patients with pollinosis (15). In addition, we demonstrated in that study that Lm-specific IgE antibodies are highly cross-reactive with pollen allergens from other grass species, but Lm-specific or cross-reactive IgE-binding components have not been identified.

In the present study, we confirmed our previous results concerning the use of the Lm extract in SPT and ELISA for the detection of IgE responses in an independent group of patients with pollinosis. We verified that conjunctivitis was observed only in SAR patients, whereas allergic rhinitis was the most frequent diagnosis in both grass pollen- and mite-sensitized patients. These findings emphasize the important role and predominant feature of conjunctivitis, associated or not with rhinitis, in the clinical symptoms of SAR patients, and show that grass pollen allergens in contact with the ocular conjunctiva and nasal mucosa can trigger symptoms related to allergic rhinoconjunctivitis in pollen-sensitized subjects (6). The SPT results were in agreement with SPT positivity between the Lm extract and mixed grass pollen extract (GII) that is routinely used for the diagnosis and immunotherapy of pollinosis in SAR patients. However, the mean wheal size of these patients was larger when using the Lm extract than the GII mix, confirming the higher sensitivity of the Lm extract in SPT by containing Lm-specific components, unlike the GII extract that contains a mix of *L. perenne* and other grass pollen allergens that are not predominant in the studied region.

In addition, it is widely known that there is a high variability in extracts of the same allergenic grass species produced by different companies, both qualitatively and quantitatively (21). Therefore, the use of a single grass species, such as the Lm extract, could be more appropriate than a mix of grass pollen allergens for the diagnosis, treatment and follow-up of patients sensitized and under immunotherapy in this geographic area.

Allergens from mites, particularly *D. pteronyssinus* and *D. farinae*, are also important sensitizing agents in regions of temperate climate (22) and in Brazil (23,24), including *B. tropicalis* that may also be an important source of allergen sensitization in tropical countries (17). In the present study, the sensitization to mite allergens was more frequent among patients who had no pollinosis (PAR_{CS} and PAR_{UDI} groups), supporting the view that SAR patients were mainly sensitized to grass pollen allergens and not to mite allergens. Also, similar rates of positive SPT to mite allergens were found between the two subgroups of PAR patients, selected in different regions of Brazil, indicating similarity in the sensitization to mite allergens in subjects living in these two distinct geographic regions.

IgE levels in response to the Lm extract, when measured by ELISA, were also higher in SAR patients than in PAR and NA subjects, supporting the usefulness of ELISA in the evaluation of IgE responses to Lm grass pollen allergens.

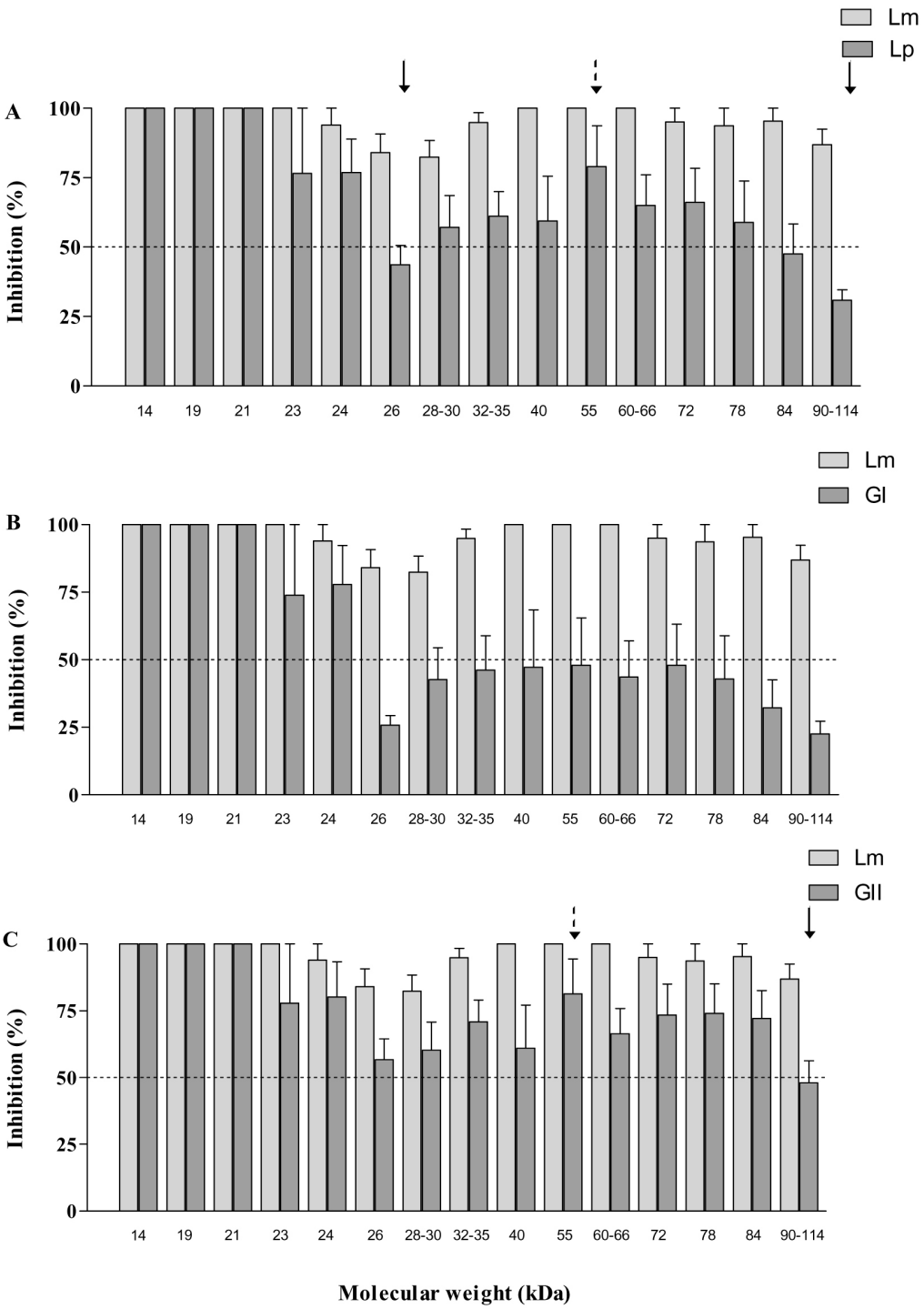

Figure 5. Inhibition (%) of IgE-binding components from the *Lolium multiflorum* (Lm) pollen extract recognized by serum samples from patients with seasonal allergic rhinitis (SAR group) after pre-adsorption with different inhibitor extracts. The dashed horizontal line indicates 50% inhibition. *A*, *L. multiflorum* and *L. perenne* (Lp). The continuous arrows indicate the least inhibited immunodominant bands and the dashed arrow indicates the most inhibited immunodominant band. *B*, *L. multiflorum* and grass mix I (GI); *C*, *L. multiflorum* and grass mix II (GII). The continuous arrow indicates the least inhibited immunodominant band and the dashed arrow indicates the most inhibited immunodominant band. Inhibitor extracts were used at the concentration of 200 µg/mL. Columns indicate the mean ± SEM percent inhibition obtained from 10 serum samples tested by immunoblot.

Studies of the characterization of the major allergens from *L. multiflorum* are limited, in contrast to a number of studies concerning the characterization, allergenic sensitization and cross-reactivity of pollen allergens from *L. perenne* and other grasses of the same family of *L. multiflorum* (1,2,10). In the present study, high homologous inhibition with low IC_{50} was observed in ELISA for the detection of IgE in response to the Lm extract, reflecting the specificity of the reaction. In addition, considerable heterologous inhibition was also noted when using Lp and GII extracts as inhibitors, indicating an extensive cross-reactivity between Lm allergens and Lp or GII pollen extracts. Since Lp allergens are present in the GII, but not in the GI extract, we may state that the IgE cross-reactivity observed when using the GII extract as inhibitor might have a substantial participation of Lp allergens, as previously suggested (15). However, in the present study, we found a lower percent heterologous inhibition with both Lp and GII extracts when compared to our previous study (15), even using higher concentrations of inhibitors. These findings can be explained by differences related to patient serum samples, which might have had different antibody levels and might have been collected in different pollen seasons. These results demonstrate that we can observe considerable heterogeneity in allergic antibody responses among different patients as well as in different seasons.

At least fifteen protein components present in the Lm extract were visualized by SDS-PAGE, similarly to a previous report that identified 14 components ranging from 14 to 80 kDa in an *L. perenne* crude extract (25). Concerning the IgE-binding Lm components, immunoblot results revealed a number of immunodominant and minor allergenic bands recognized by SAR patients. The major components recognized by IgE (>90% recognition) had apparent molecular masses of 26, 28-30, and 32-35 kDa, the latter two probably corresponding to allergens from groups 1 and 5, since group 1 consists of glycoproteins of 32-35 kDa, and group 5 of non-glycosylated proteins with 28-30 kDa (26,27). In the Lm extract, antigenic components of 55 and 60-66 kDa were also recognized by IgE antibodies of most patients with pollinosis. These components could correspond to allergens from groups 4 and 13, as previously reported in several clinical studies demonstrating the importance of grass pollen allergens from these groups (13-15). Accordingly, in the *L. perenne* pollen extract, Lol p 4 was characterized as a protein of 59 kDa (28), whereas in the *P. pratense* pollen extract, Phl p 4 was identified with a molecular mass of 55 kDa (13). Despite the similar molecular mass range of grass pollen allergens from groups 4 and 13, isoelectric focusing followed by two-dimensional immunoblot showed that they have different isoelectric points (pI), as described for Phl p 4, a glycoprotein with a pI of 9.4, and Phl p 13, a glycoprotein with a pI of 7.5 (13,14).

In our study, the high frequency of IgE reactivity to 90-114-kDa components from *L. multiflorum* suggests their clinical importance and a possible relation with a high-molecular weight basic allergen of *L. perenne* pollen previously characterized immunochemically (28). In addition, an antigenic component of 100 kDa of *Cynodon dactylon*, a Chloridoideae subfamily grass present in Southern Brazil, was also considered to be clinically important by its recognition in the majority of patients with seasonal allergic rhinitis (29). Thus, the band of 90-114 kDa of *L. multiflorum* pollen extract could present IgE cross-reactivity with components of high molecular weight of *C. dactylon* (28,29), but studies using specific monoclonal antibodies and inhibition immunoassays should be conducted to clarify this issue.

The Lm extract antigenic component of low molecular mass (14 kDa) recognized by 42% of SAR patients of this study can be related to grass pollen allergens from groups 2, 3, 10, or 11, which have a molecular mass ranging from 10 to 16 kDa. Allergens from groups 2 and 3 were previously identified as proteins of approximately 10-12 kDa (30), showing different pI, an acid one and a basic one, when separated on two-dimensional gel and even quantified by using monoclonal antibody techniques (31), while allergens from groups 10 (≅12 kDa) and 11 (≅16 kDa) can also be distinguished by their different pI (10 and 5-6, respectively) (12,32).

In the inhibition immunoblot performed here, a high homologous inhibition rate (>80%) was observed in almost all antigenic bands, including the immunodominant ones. The Lp and GII inhibitor extracts showed more than 50% inhibition for nearly all immunodominant components of the Lm extract, except the 26- and 90-114-kDa bands. As these components showed high homologous, but weak heterologous inhibition, it appears that they are specific antigenic components of *L. multiflorum*.

Pollen allergens from groups 1 and 5 are highly conserved in most clinically relevant grasses (33-36) and proved to be important in this study. However, the 28-30- and 32-35-kDa components likely related to allergens from groups 5 and 1, respectively, showed more than 50% inhibition with the Lp and GII extracts, and less than 50% inhibition when the GI extract was used. Thus, there was a limited IgE cross-reactivity between the Lm and GI extracts, in contrast to an extensive epitope sharing between the Lm and Lp or GII mix extracts. These findings can be explained by the phylogenetic proximity of *L. multiflorum* and *L. perenne* or grasses present in the GII extract (10). Accordingly, a recent study showed a complete lack of inhibition between the Bermuda grass group 1 allergen rCyn d 1 and the rye grass group 5 allergen rLol p 5, showing that the limited cross-reactivity between the rye and Bermuda grasses may be due, in part, to the absence of the group 5 allergen in the latter (37). Among the immunodominant components, the band of 55 kDa showed the highest heterologous inhibition (approximately 80%) when using both Lp and GII extracts, suggesting that this Lm antigenic component, likely related to allergens from groups 4 or 13, may represent

an important IgE cross-reactivity among allergens from *L. multiflorum* pollen and other grasses. It is noteworthy that *L. multiflorum* allergens are not included in the Lp and GII extracts, although there is a great phylogenetic proximity between *L. multiflorum* and grasses present in those extracts. In contrast, the GI extract, which showed less than 50% inhibition for Lm immunodominant components, presents in its composition grass pollen tribes (Triticeae and Aveneae) phylogenetically different from *L. multiflorum* (Poeae). Altogether, these results are consistent with inhibition radioimmunoassay and ELISA studies, using crude extracts of grass pollen of the family Poaceae that showed strong cross-reactivity between their members (1,10,36). Further inhibition experiments should be conducted with purified or recombinant major allergens to confirm the grouping of the individual allergens.

The present study showed that immunodominant components of *L. multiflorum* pollen present high IgE cross-reactivity with allergens from *L. perenne* pollen and grass pollens present in the GII extract, particularly the band of 55 kDa. Therefore, IgE cross-reactivity among grass pollen allergens should be evaluated in the formulation of allergenic extracts for diagnostic purposes or for allergen-specific immunotherapy in order to avoid inadvertent adverse reactions, especially in extract mixes containing pollens from various species of grasses, resulting in high concentrations of conserved allergens (10). In addition, specific *L. multiflorum* allergens, particularly components of 26 and 90-114 kDa, should be further characterized concerning their real cross-reactivity with pollen of other grasses for diagnostic use and for allergen-specific immunotherapy.

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