

ORIGINAL ARTICLE Allergens

Functional immunoglobulin E cross-reactivity between Pas n 1 of Bahia grass pollen and other group 1 grass pollen allergens

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Summary

Background Grass pollens are major triggers of allergic rhinitis and asthma, but the immunological relationships between pollen allergens of the subtropical Bahia grass, *Paspalum notatum*, and temperate grasses are unresolved.

Objective To assess serum IgE cross-reactivity between subtropical *P. notatum* and temperate *Lolium perenne* (Ryegrass) pollen allergens.

Methods Serum IgE reactivities of grass pollen-allergic patients with *P. notatum*, *L. perenne* and *Cynodon dactylon* (Bermuda grass) pollen extracts and their respective purified group 1 allergens, Pas n 1, Lol p 1 and Cyn d 1, were compared by immunoblotting, ELISA and basophil activation.

Results In a cohort of 51 patients from a temperate region, a high frequency of IgE reactivity with each grass pollen was detected, but reactivity with *L. perenne* pollen was substantially greater than with *P. notatum* and *C. dactylon* pollen. Similarly, serum IgE reactivity with Lol p 1 was greater than with Pas n 1 or Cyn d 1. For seven of eight sera studied in detail, asymmetric serum IgE cross-reactivity was observed; *L. perenne* pollen inhibited IgE reactivity with *P. notatum* pollen but not the converse, and IgE reactivity with Pas n 1 was inhibited by Lol p 1 but IgE reactivity with Lol p 1 was not inhibited by Pas n 1 or Cyn d 1. Importantly, *P. notatum* pollen and Pas n 1 activated basophils in grass pollen-allergic patients from a temperate region, although stimulation was greater by pollen of *L. perenne* than *P. notatum* or *C. dactylon*, and by Lol p 1 than Pas n 1 or Cyn d 1. In contrast, a cohort of 47 patients from a subtropical region showed similar IgE reactivity with *P. notatum* and *L. perenne* pollen, and reciprocal cross-inhibition of IgE reactivity between *L. perenne* and *P. notatum*.

Conclusions Pollen allergens of the subtropical *P. notatum*, including Pas n 1, show clinically relevant IgE cross-reactivity with pollen allergens of *L. perenne* but also species-specific IgE reactivity.

Keywords allergic rhinitis, Bahia grass pollen, grass pollen allergy, IgE cross-reactivity, Pas n 1, Ryegrass pollen

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Introduction

Grass pollen allergens are highly recognized aeroallergens world-wide for patients with seasonal allergic diseases including allergic rhinitis (AR) and asthma [1]. Air-borne grass pollen levels are associated with hospital admissions for asthma [2]. The prevalence of AR and asthma has increased drastically in the developed world over recent

decades and grass pollen allergens are major culprits [1, 3–5]. AR has serious clinical consequences *per se* but confers additional adverse effects for asthmatics [6].

Administration of grass pollen immunotherapy in children with AR reduced the onset of asthma in school-age children in whom there was a high risk of subsequent asthma [7, 8]. Recently, tablets for sublingual immunotherapy (SLIT) for grass pollen allergy were licensed

enabling improved quality control, as well as convenient prescription and acquisition of immunotherapy treatment by patients [9]. These SLIT grass pollen tablets are based on a whole pollen extract of *Phleum pratense* (Timothy grass), a clinically important allergenic grass derived from the Pooideae subfamily of temperate grass species [10, 11]. Although *P. pratense* extract is considered by many to be largely representative of grass pollen allergens, debate continues as to whether a single allergenic extract of a temperate grass pollen endemic in parts of the northern hemisphere is sufficient to effectively tolerate allergic responses to all grass pollen allergens [12–15]. Other SLIT tablets or solutions based on mixes of five temperate grass species are also effective in clinical trials for treatment of grass pollen allergy [16–18], but these still lack representation of grass pollens outside the Pooideae subfamily of grasses. Allergenic molecules derived from subtropical grass species differ significantly in terms of allergenic components, primary amino acid sequences of common allergenic molecules and immunological reactivity [19, 20].

Cross-reactivity between pollen allergens of temperate grasses is well established [21–23]. Furthermore, the immunological relationship between temperate grass pollen allergens and subtropical grass pollens of the Chloridoideae subfamily represented by *Cynodon dactylon* (Bermuda grass) has previously been described [24, 25]. However, there is limited understanding of clinical and immunological cross-reactivity of subtropical grasses of the Panicoideae subfamily represented by *Paspalum notatum* (Bahia grass) with other grass pollen allergens. *P. notatum* is distributed widely in urban areas including temperate regions. It has a prolonged period of pollination and is reported to trigger AR late in the pollen season in temperate regions [26, 27]. Given the phylogenetic distinction of *P. notatum* from temperate grass species, including the clinically important temperate grasses, *P. pratense* and *Lolium perenne* (perennial Ryegrass), the capacity of *P. notatum* pollen allergens for immunological cross-reactivity with temperate grass pollen allergens warrants further investigation.

We recently cloned and characterized the group 1 allergen of Bahia grass pollen, Pas n 1 [19]. Pas n 1 is a major allergen of *P. notatum* pollen, reactive with serum IgE of 85% grass pollen-allergic patients in a temperate zone and with potent capacity to activate basophils from grass pollen-allergic donors [19]. Consistent with the phylogenetic relationship between grasses, Pas n 1 has greatest sequence identity with Zea m 1 of maize, *Zea mays* (85%), another Panicoideae subfamily member, and lower identity with Pooideae group 1 pollen allergens of *L. perenne* (Lol p 1, 64%) and *P. pratense* (Phl p 1, 66%) [19, 28, 29]. Cyn d 1 of *C. dactylon*, a Chloridoideae subfamily species, has 67% identity with Pas n 1. These homologies are sufficient to suggest cross-recognition of the group 1 allergens from these grasses by patient serum IgE [19, 30, 31], but IgE cross-reactivity of *P. notatum* pollen allergens

with allergens of other grass pollens has not previously been defined at a molecular level. Here, we compare serum IgE reactivity with *P. notatum*, *C. dactylon* and *L. perenne* pollen extracts in a cross-section of grass pollen-allergic patients from temperate and subtropical regions and investigate in detail serum IgE cross-reactivity in individual patients.

Materials and methods

Patients

Sera were obtained from a panel of 51 patients living in a temperate region presenting with AR to the Alfred Hospital Allergy Clinic, Melbourne, Victoria, Australia, and with positive skin prick test (SPT), ELISA and/or ImmunoCAP (Phadia, Uppsala, Sweden) for IgE reactivity with *L. perenne* and *P. notatum* pollen [19]. Sera from healthy, non-atopic controls ($n = 15$) who were negative by SPT to a panel of common environmental aeroallergens including *L. perenne* and *P. notatum* pollen and atopic non-grass pollen-allergic donors ($n = 12$) were also collected. Approval was obtained from the Alfred Hospital Ethics Committee with informed written consent from each subject. Plasma was obtained from a panel of 47 donors, with sera from five of these donors, from Brisbane, Queensland, Australia, who were sensitized to grass pollen allergens with informed consent as approved by the Princess Alexandra Hospital Human Research Ethics Committee.

Allergen extracts and purified allergens

Non-defatted extracts of *L. perenne*, *P. notatum*, *P. pratense* and *Sorghum halepense* and *C. dactylon* pollen (Greer Laboratories, Lenoir, NC, USA) were prepared in phosphate-buffered saline [27]. Roasted peanut extract (RPN) was prepared as a control allergen [32]. Group 1 allergens Pas n 1, Lol p 1 and Cyn d 1 were purified from pollen extracts of *P. notatum*, *L. perenne* and *C. dactylon*, respectively, by ammonium sulphate precipitation, hydrophobic interaction and size exclusion chromatography as described by Drew *et al.* [33]. The purity and identity of Pas n 1, Lol p 1 and Cyn d 1 were confirmed by one- and two-dimensional gel electrophoresis visualized by Coomassie brilliant blue (CBB) staining as well as immunoblotting with allergen-specific monoclonal antibodies. Lol p 1 and Pas n 1 were detected using a monoclonal antibody specific for Lol p 1, FMC-A1 [34], which also reacts with Pas n 1. A monoclonal hybridoma-producing anti-Cyn d 1 antibody (6/C6) of the IgG₁ subclass was generated as previously described [35] and used to detect Cyn d 1. The concentration of extracts and purified allergens was determined using the Coomassie dye binding assay with bovine γ -globulin as a standard (BioRad, Hercules, CA, USA).

Immunoglobulin reactivity with grass pollen extracts and purified group 1 allergens

ELISAs were performed to determine the level, titre and frequency of IgE reactivity with whole grass pollen extracts (10 µg/mL) and purified group 1 allergens (1 µg/mL) as described previously [27]. Gaussian approximation of *P*-values for difference in median IgE reactivity was calculated by a two-tailed Wilcoxon signed rank paired test. For donors from a subtropical region, plasma diluted 1/10 were tested in ELISA with microtitre plate wells coated with 5 µg/mL of whole grass pollen extract.

Inhibition of serum immunoglobulin E reactivity with *Paspalum notatum* and *Lolium perenne* pollen allergens by grass pollen extracts and group 1 allergens

Inhibition assays were conducted for individual sera diluted to a concentration within the linear phase of titration curves of IgE reactivity with *L. perenne* or *P. notatum* pollen at approximately 1 OD unit. Inhibitor grass pollen allergen extracts (0.05–100 µg/mL) or purified Pas n 1, Lol p 1 and Cyn d 1 (0.01–10 µg/mL) were pre-incubated with diluted sera for 90 min before addition in triplicate to antigen-coated and blocked microtitre plate wells [27]. The median and interquartile range of maximum percentage inhibition values observed for the grass pollen extracts and allergens on IgE reactivity with *P. notatum* and *L. perenne* pollen of eight sera were determined.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotting

Grass pollen extract proteins, 20 µg/lane, were separated under reducing conditions by 12% SDS-PAGE and stained

with CBB. Immunoblot assays for serum IgE reactivity with separated proteins were conducted as described previously [27].

Basophil activation assay

L. perenne, *P. notatum* and *C. dactylon* pollen extracts as well as Pas n 1, Lol p 1 and Cyn d 1 were tested from 0.005 to 5 µg/mL for activation of basophils from whole blood, based on surface expression of CD63 on live cells gated on high IgE-positive basophils by flow cytometry [36]. Ovalbumin served as a negative control antigen (Sigma Aldrich, St Louis, MO, USA).

Results

Comparative serum immunoglobulin E reactivity with grass pollen extracts

The patterns of IgE reactivity with pollen extracts of *P. notatum* and *L. perenne* were examined by immunoblotting with sera from five patients presenting with grass pollen allergy in a tertiary allergy clinic in a temperate region (Fig. 1a). IgE reactivity with *L. perenne* pollen was directed against protein bands consistent with the group 1 (33 kDa) and 5 (28 and 30 kDa) allergen bands in four of the five sera and against a 55 kDa band in two sera. Four sera reacted with both the group 1 allergen (29 kDa) and the 55 kDa band of *P. notatum* pollen. No serum reacted with bands equivalent to a group 5 allergen in *P. notatum* pollen (Fig. 1a, and data not shown for an additional 30 sera tested). Protein bands consistent with the group 1 allergen were the major reactant for serum IgE in both extracts.

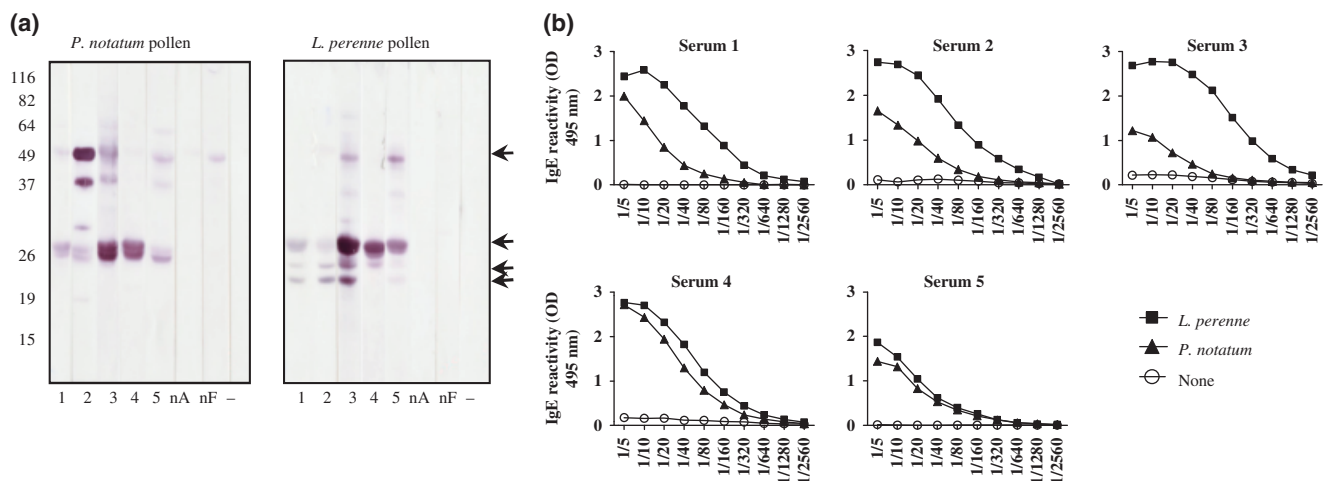


Fig. 1. Comparative serum IgE reactivity with *Paspalum notatum* and *Lolium perenne* in patients from a temperate region. (a) IgE immunoblots of *L. perenne* and *P. notatum* pollen extracts probed with grass pollen-allergic patients (1–5) and non-atopic donor sera (nA and nF; ‘-’ no serum; sizes in kDa). Arrows indicate allergen positions; high molecular weight allergen (55 kDa), group 1 (~29 kDa) and group 5 in *L. perenne* (28 kDa doublet). (b) Titration of serum IgE reactivity with pollen of *L. perenne* and *P. notatum* by ELISA.

These five sera examined by immunoblotting were investigated by titration ELISA. The levels and titres of IgE reactivity with *P. notatum* pollen were substantially lower than with *L. perenne* pollen for three of the five sera tested (Fig. 1b). Sera four and five showed similar level and titre of IgE reactivity with *L. perenne* and *P. notatum* pollen.

Apparent differences between the intensity of bands visualized on IgE immunoblots and the level of IgE reactivity by ELISA (e.g. serum 1) might be accounted for by the different assay formats whereby some conformational B cell epitopes may be disrupted by immunoblotting.

Serum immunoglobulin E reactivity of a panel of patients from temperate region with grass pollen extracts and group 1 allergens Pas n 1, Lol p 1 and Cyn d 1

In a larger cross-sectional panel of 51 sera derived from grass pollen-allergic patients from a temperate region, the frequency of IgE reactivity with pollen of *L. perenne*, *P. notatum* and *C. dactylon* was equally high (90–92%). However, the median level of IgE reactivity was significantly higher with pollen extract of *L. perenne* than with *P. notatum* and *C. dactylon*. Serum IgE reactivity did not differ significantly between the two subtropical grass pollen extracts (Fig. 2a). Serum IgE reactivity for the same panel of patients with the group 1 allergens purified from *L. perenne*, *P. notatum* and *C. dactylon* was greatest with Lol p 1. The median level of IgE reactivity with Pas n 1 was higher than with Cyn d 1 (Fig. 2a).

To confirm the differences in observed serum IgE reactivity with the various grass pollens and their group 1 allergens, three individual sera (6–8) were titrated simultaneously against whole pollen extracts of *L. perenne*, *P. notatum* and *C. dactylon* as well as Lol p 1, Pas n 1 and Cyn d 1 (Fig. 2b). Consistent with the cross-sectional study (Fig. 2a) and data in Fig. 1, these individual sera showed higher levels and titres of IgE reactivity with pollen of *L. perenne* than with *P. notatum* or *C. dactylon*. The level and titre of IgE reactivity was higher in these individual serum donors with Lol p 1 compared with Pas n 1 and Cyn d 1 (Fig. 2b).

Inhibition of serum immunoglobulin E reactivity with Lolium perenne and Paspalum notatum by grass pollen extracts

We investigated whether the one-way cross-inhibition between *L. perenne* and *P. notatum* observed previously for IgE reactivity of pooled sera [27] would be evident in individual sera, in particular the sera (4 and 5) with similar levels and titres of IgE reactivity with both of these grasses. For the eight sera tested (including those from Figs 1 and 2b), IgE reactivity with pollen of *L. perenne* was inhibited by pollens of both temperate grasses *L. perenne* and *P. pratense*, but not by subtropical *P. notatum*,

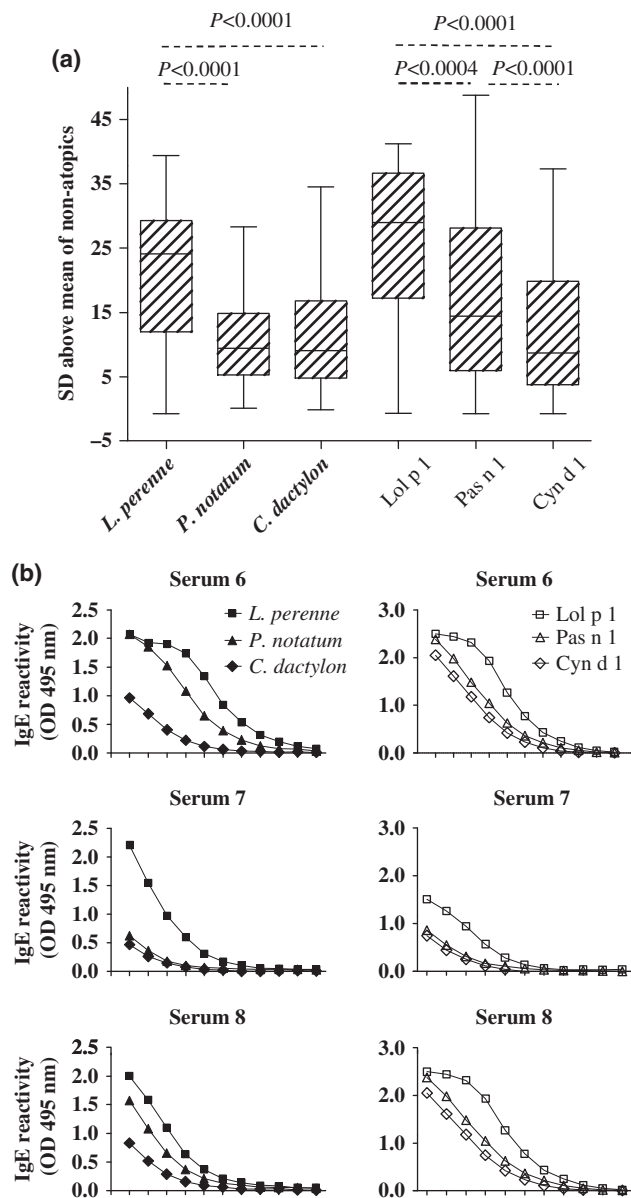


Fig. 2. Serum IgE reactivity in a panel of grass pollen-allergic donors from a temperate region with whole pollen extracts and purified group 1 allergens. (a) Box and whisker plots showing median and distribution (25–75 percentiles) of IgE reactivity of 51 grass pollen-allergic donor sera with grass pollen extracts and purified group 1 allergens (P -values given for Wilcoxon signed rank paired test). (b) Titration of serum IgE reactivity of grass pollen-allergic patients with grass pollen extracts and purified group 1 allergens.

S. halepense and *C. dactylon* grass pollens (Table 1). In the converse experiments, IgE reactivity with pollen of *P. notatum* was inhibited by *P. notatum* as well as by *L. perenne*, *P. pratense*, *S. halepense* and partly by *C. dactylon* (Table 1). Serum 3 exemplifies data observed for seven of eight sera showing the inhibition of IgE reactivity with *L. perenne* by the grass pollen extracts where only *L. perenne* and *P. pratense* were inhibitory

Table 1. Inhibition of IgE reactivity with *Lolium perenne* and *Paspalum notatum* by temperate and subtropical grass pollen extracts

Serum	<i>L. perenne</i>	<i>P. pratense</i>	<i>P. notatum</i>	<i>S. halepense</i>	<i>Cynodon dactylon</i>	Roasted peanut extract	None
Coating antigen: <i>L. perenne</i>							
1	99 (0.5)*	90 (5)	13	6	-16	16	0
2	97 (0.4)	89 (11)	1	7	3	1	0
3	97 (0.3)	70 (20)	9	7	7	4	0
4	98 (<0.1)	89 (0.8)	74 (4)	68 (10)	47	-5	0
5	96 (0.9)	75 (15)	16	10	20	3	0
6	95 (0.8)	88 (2)	20	24	25	8	-2
7	85 (20)	64 (70)	6	2	5	4	-3
8	85 (1)	82 (0.1)	25	30	18	13	3
Median	96.5	85	14.5	8.5	12.5	4	0
IQR [†]	92-97	73-89	8-21	7-25	6-21	2-9	-0.5 to 0
Coating antigen: <i>P. notatum</i>							
1	80 (<0.1)	78 (0.6)	86 (2.5)	72 (9)	53 (90)	41	0
2	42	40	63 (20)	43	38	32	0
3	95 (0.1)	93 (1)	86 (8.5)	83 (9.5)	54 (75)	18	0
4	95 (<0.1)	95 (0.1)	96 (<0.1)	86 (0.9)	80 (0.3)	-6	0
5	58 (13)	66 (12)	92 (0.6)	79 (3)	60 (0.6)	18	0
6	94 (1)	94 (1)	100 (0.8)	57 (5)	60 (5)	22	0
7	94 (0.6)	95 (0.9)	96 (0.5)	81 (7)	53 (85)	2	0
8	86 (0.5)	78 (3)	90 (0.1)	78 (7)	58 (55)	36	0
Median	90	85.5	91	78.5	56	20	0
IQR	79-94	75-94	89-96	72-81	54-60	14-33	0-0

*Inhibition results given as maximum percentage inhibition. The concentration giving 50% inhibition (IC_{50}) from inhibition dose-response curves is given in parentheses ($\mu\text{g/mL}$). No IC_{50} value indicates that 50% inhibition was not achieved at 100 $\mu\text{g/mL}$.

[†]Interquartile range (IQR) and median maximum inhibition values are given.

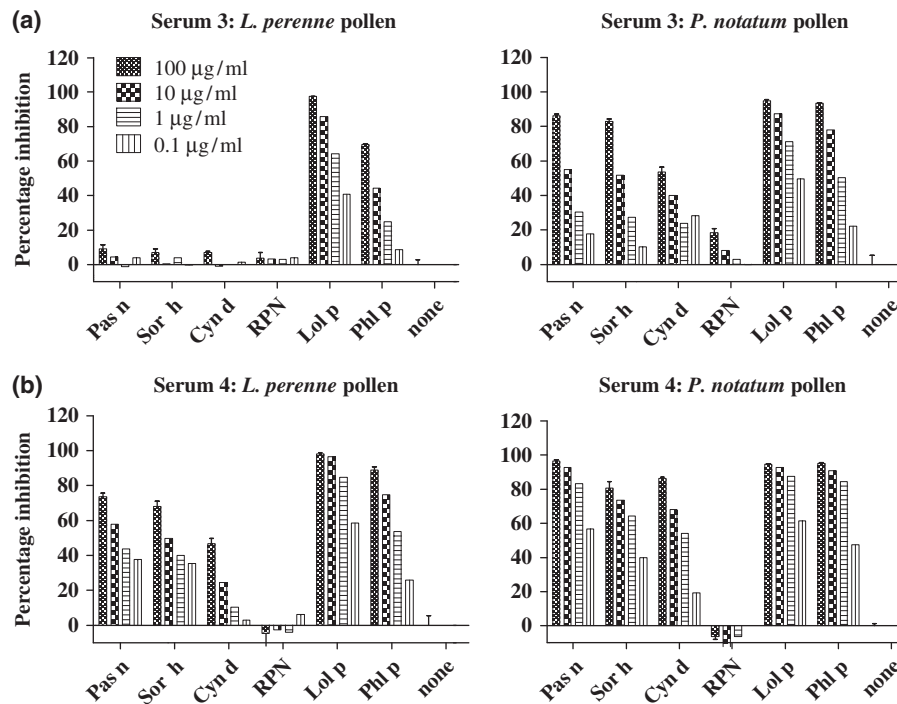


Fig. 3. Cross-inhibition of IgE reactivity of sera 3 (a) and 4 (b) with pollen extract of *Lolium perenne* and *Paspalum notatum* by grass pollen and roasted peanut extract extracts (0.1–100 $\mu\text{g/mL}$) as listed on the x-axis.

(Fig. 3a). In contrast, IgE reactivity with pollen of *P. notatum* was inhibited by not only the subtropical grass pollens *P. notatum*, *S. halepense* and to a lesser extent

C. dactylon pollen, but also by temperate grass pollens *L. perenne* and *P. pratense* (Fig. 3a). Notably for serum 4, which had a similar level and titre of IgE reactivity with

both *P. notatum* and *L. perenne* pollen extracts, IgE reactivity with *L. perenne* was inhibited by *L. perenne* and *P. pratense* but also by *P. notatum*, *S. halepense* and *C. dactylon* (Fig. 3b). For serum 4, IgE reactivity with pollen of *P. notatum* was inhibited by *P. notatum*, *S. halepense*, *C. dactylon* as well as *L. perenne* and *P. pratense*, demonstrating partial reciprocal cross-inhibition. For patient 5, who also had a similar titre of IgE reactivity with *P. notatum* and *L. perenne* but higher level IgE reactivity with *L. perenne* by ELISA and immunoblotting, IgE reactivity with *L. perenne* was not inhibited by *P. notatum* (Table 1). Notably, serum 5 IgE reactivity with *P. notatum* was not completely inhibited by *L. perenne* and *P. pratense*, suggesting that *P. notatum* contained unique B cell epitopes recognized by this serum that were not present in the temperate grass pollens.

A low avidity interaction between serum IgE and epitopes of *P. notatum* pollen, and the presence of plant panallergens like profilins or cross-reactive carbohydrate moieties could account for the partial inhibition of IgE reactivity with *P. notatum* by 100 µg/mL of RPN observed in four of eight sera (maximum percentage inhibition 22–41%; Table 1). However, inhibition was not seen at lower concentrations of RPN and in these same sera, RPN failed to inhibit IgE reactivity with *L. perenne* pollen.

Inhibition of serum immunoglobulin E reactivity with Pas n 1 and Lol p 1

Three representative sera typical of the panel of 51 grass pollen-allergic patients from a temperate climate (sera 6–8) and serum 4, which had similar IgE reactivity with *L. perenne* and *P. notatum* pollen and had shown reciprocal cross-inhibition with whole pollen extracts, were tested for cross-inhibition of IgE reactivity with Lol p 1 and Pas n 1 (Fig. 4a). IgE reactivity with Pas n 1 was inhibited by Pas n 1, Lol p 1 and partly by Cyn d 1. In contrast, IgE reactivity with Lol p 1 was not inhibited by Pas n 1 or Cyn d 1 except in serum 4, which showed up to 60% and 40% inhibition by Pas n 1 and Cyn d 1, respectively. The asymmetric inhibition observed for sera 6–8 indicates that the avidity of IgE interaction with Lol p 1 in these sera was greater than between IgE and Pas n 1 or Cyn d 1. Only for serum 8 was there good concordance of inhibition of IgE reactivity with Pas n 1 by Pas n 1 and Lol p 1, suggesting that this was the only serum that did not show IgE reactivity with unique Pas n 1 epitopes.

Basophil activation by grass pollen extracts and purified group 1 allergens

Whole pollen extracts of *L. perenne* as well as *P. notatum* and *C. dactylon* potentially activated basophils of six grass pollen-allergic donors from a temperate region (Fig. 5). There was a trend that pollen of *L. perenne* stimulated

basophils at lower concentrations than pollen of *P. notatum* and *C. dactylon*. Lol p 1 as well as Pas n 1 and Cyn d 1 activated basophils of these grass pollen-allergic donors. Similar to the whole pollen extracts, Lol p 1 stimulated basophils at lower concentrations than Pas n 1 and Cyn d 1. In patients 6, 44 and 45, a bell-shaped dose-response curve was observed for basophil activation, consistent with over-stimulation of the basophils at high concentrations of allergen and the high sensitivity of these patients to grass pollen. Basophils of a non-atopic donor were not activated by any of the allergens but were activated by the positive controls fMetLeuPhe peptide (53.4%) and anti-IgE cross-linking (82.0%) (data not shown).

Immunoglobulin E reactivity with grass pollen allergens in patients from a subtropical climate

A panel of 47 grass-pollen-allergic patients living in a subtropical region did not show a dominance of serum IgE reactivity with *L. perenne* pollen (Fig. 6a). There was a trend for higher plasma IgE reactivity with *P. notatum* pollen compared with *L. perenne* pollen but the difference was not significant. For three of five individual patients from a subtropical region, the titre and level of IgE reactivity was higher with *P. notatum* than *L. perenne* grass pollen (Fig. 5b). By IgE immunoblotting, the serum IgE reactivity with *P. notatum* pollen of patients from a subtropical region showed stronger bands consistent with the high molecular weight allergen doublet (55 and 50 kDa) and with a band corresponding to the group 1 allergen (29 kDa) (Fig. 6c). Serum 183 did not show IgE reactivity with either pollen extract by immunoblotting. Consistent with the titration ELISA data (Fig. 6b), sera 56 and 188 showed stronger bands by IgE immunoblotting of *P. notatum* pollen than *L. perenne* pollen (Fig. 6c). A representative serum from a temperate region (number 45) tested in the same assay confirmed the data of Fig. 1a with IgE reactivity with *L. perenne* pollen allergens consistent with the group 1 (30 kDa), group 5 allergens (26 and 28 kDa) as well as the 55 kDa allergen. This serum only showed weak IgE reactivity with high-molecular-weight allergen bands of *P. notatum* pollen. Inhibition ELISA with four individual sera from a subtropical region showed reciprocal cross-inhibition of IgE reactivity with *P. notatum* and *L. perenne* pollen extracts (Fig. 6d). The inhibition of IgE reactivity with *P. notatum* pollen was stronger by subtropical grass pollen extracts than by temperate grass pollen extracts with the maximum percentage inhibition of 90.5% by *P. notatum* compared with 64.5% by *L. perenne* pollen.

Discussion

The allergenic relationship between *P. notatum* pollen and other clinically important grass pollens has hitherto been

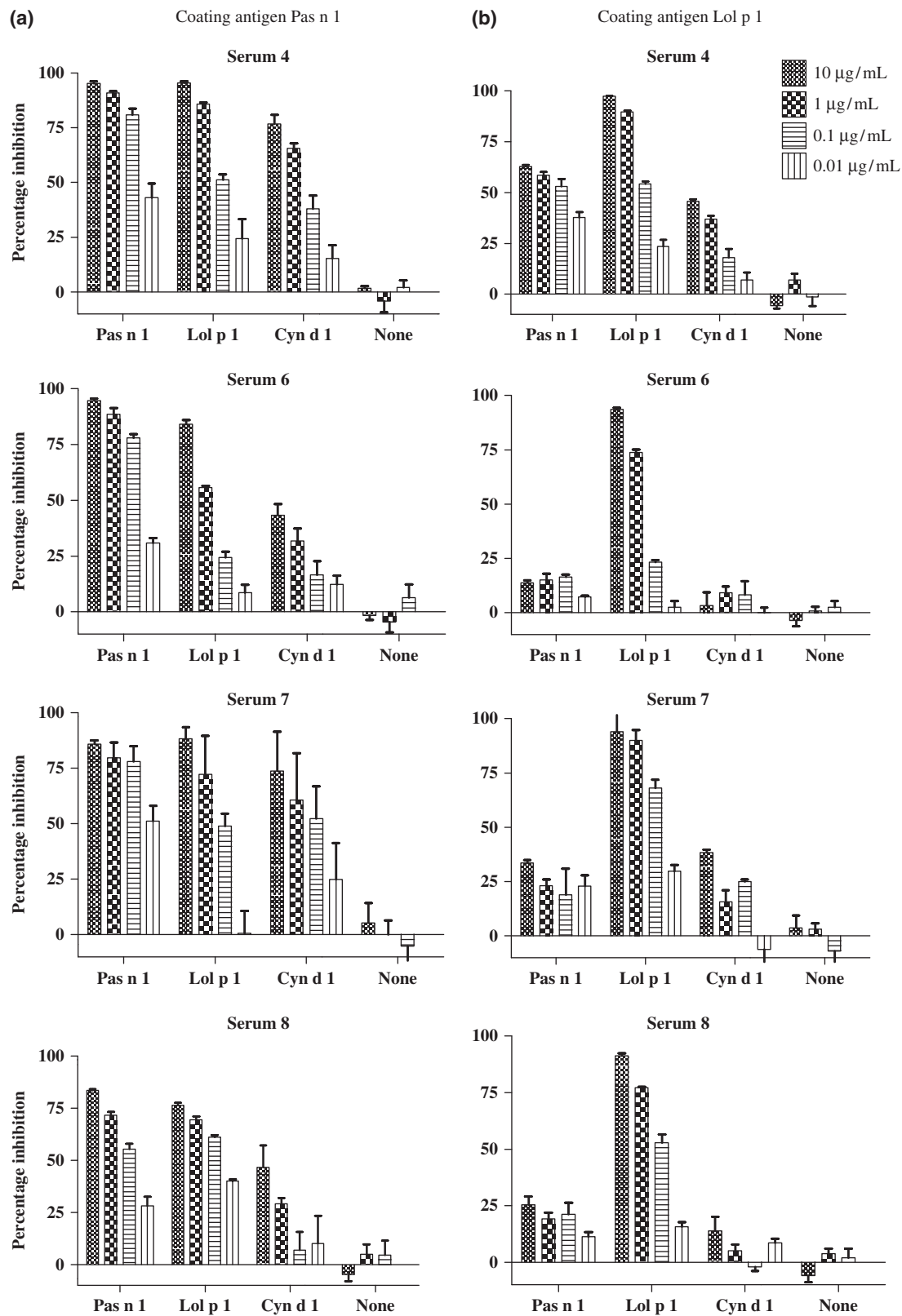


Fig. 4. Cross-inhibition of IgE reactivity with Pas n 1 (a) and Lol p 1 (b) by purified group 1 allergens at 0.01–10 µg/mL as listed on the *x*-axis.

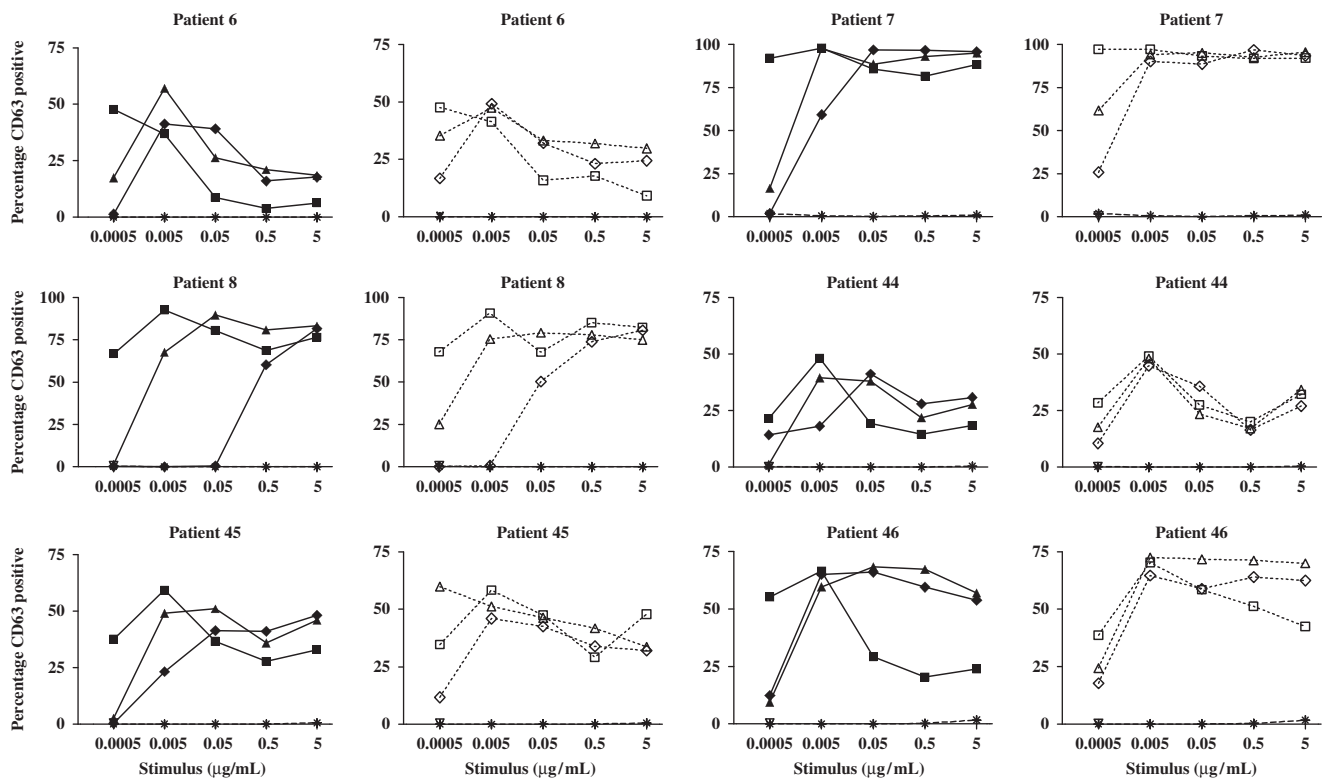


Fig. 5. Activation of basophils of grass pollen-allergic donors from a temperate region by grass pollen extracts and purified group 1 allergens (■, *Lolium perenne*; ▲, *Paspalum notatum*; ◆, *Cynodon dactylon*; □, Lol p 1; △, Pas n 1; ◇, Cyn d 1).

unclear. This issue has significant implications for exacerbation of AR as well as for accurate diagnosis and optimal specific immunotherapy for grass pollen allergy. Although in patients from a temperate region, *P. notatum* pollen and its major allergen Pas n 1 showed lower serum IgE reactivity than *L. perenne* pollen and asymmetric cross-reactivity with *L. perenne* pollen, this reactivity is clinically relevant as demonstrated by potent activation of basophils by *P. notatum* pollen and its major allergen Pas n 1.

Our data on patients from a temperate region fit with the majority of patients in this study living in a region where *L. perenne* is abundant but where other grasses including *P. notatum* and *C. dactylon* are also found. The higher level and titre of IgE reactivity with *L. perenne* pollen compared with *P. notatum* pollen is consistent with higher IgE ImmunoCAP levels for *L. perenne* than *P. notatum* pollen.

Although studies comparing IgE reactivity between pollen allergens of *C. dactylon* and temperate grasses, *P. pratense* and *L. perenne*, have revealed antigenic differences [22, 23], serum IgE reactivity between *P. notatum* and *C. dactylon* and their respective allergens has not previously been directly compared. No previous studies show individual serum IgE cross-inhibitions, comparative serum IgE titrations or basophil activation studies with *L. perenne* and *P. notatum* pollen and their purified group 1 allergens. Here pollen extracts of the two subtropical grass pollens *P. notatum* and *C. dactylon* showed similar levels of IgE

reactivity in patients from a temperate region. However, *C. dactylon* showed less inhibition of IgE reactivity of *P. notatum* than *P. notatum* or *S. halepense* and tended to activate basophils less than *P. notatum*, consistent with antigenic differences among these subtropical grass pollens.

Asymmetric cross-reactivity suggests lower avidity interaction of serum IgE with *P. notatum* pollen allergens in patients primarily sensitized with *L. perenne* pollen [37], rather than patients multiply sensitized with distinct allergens. Theoretically, if antibodies to two unrelated antigens coexist, then the level of antibody to one antigen should not interfere with the interaction between another antibody and its antigen. In this scenario, no inhibition rather than one-way inhibition should occur. If there were not an excess of *L. perenne* pollen-specific IgE relative to *P. notatum* pollen-specific IgE in a serum sample, could the IgE reactivity with *L. perenne* pollen be inhibited by *P. notatum* pollen? In patient 4 with similar level and titre of serum IgE reactivity with pollen of both grasses, *P. notatum* and *C. dactylon* partly inhibited IgE reactivity with *L. perenne* pollen, indicating the presence of B cell epitopes shared between these grass pollens as well as species-specific IgE reactivity.

Allergen cross-reactivity is largely dependent on the presence of related allergen families within allergen extracts [31]. We noted differences between the temperate and subtropical grass pollens in terms of their allergenic

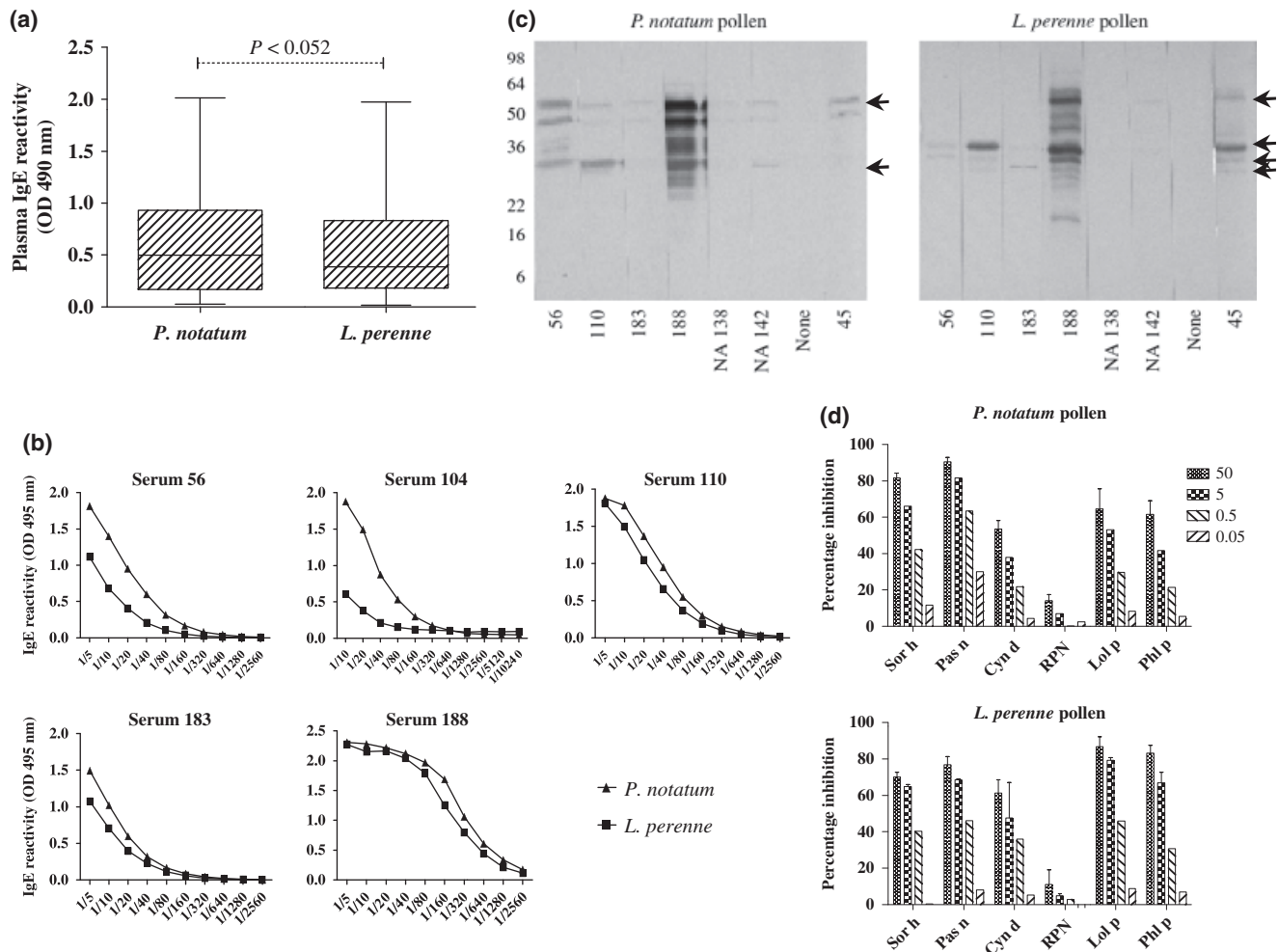


Fig. 6. IgE reactivity of grass pollen-allergic patients living in a subtropical region. (a) Box and whisker plots showing median and distribution (25–75 percentiles) of IgE reactivity of 47 grass pollen-allergic plasma donors with pollen extracts (P -values given for Wilcoxon paired test). (b) Titration of serum IgE reactivity with *Lolium perenne* and *Paspalum notatum* pollen extracts by ELISA. (c) IgE immunoblots of *L. perenne* and *P. notatum* pollen extracts probed with sera from grass pollen-allergic patients from a subtropical region (56, 110, 183 and 188), two non-atopic donors (NA138, NA142), and one grass pollen-allergic donor from a temperate region (45); sizes in kDa. Arrows indicate allergen positions; high-molecular-weight allergen bands (55 and 50 kDa), group 1 (~29 kDa) and group 5 in *L. perenne* (28 kDa doublet). (d) ELISA for inhibition of IgE reactivity with *P. notatum* and *L. perenne* pollen by grass pollen extracts at 0.05–50 µg/mL. Data presented as median and 75th percentile of inhibition values for four sera from a subtropical region (56, 110, 183 and 188).

components and in the allergen recognition by different individuals. We observed no IgE reactivity with protein bands equivalent to a group 5 allergen in *P. notatum* consistent with our earlier study [27] and with the lack of group 5 allergens outside the Pooideae subfamily of grasses [38]. The 55 kDa allergen band appears more abundant in *P. notatum* pollen than in *L. perenne* pollen. The high-molecular-weight allergen of *P. notatum* pollen is likely to be a group 13 allergen [39].

Although pollens of the temperate grasses *L. perenne* and *P. pratense* behaved similarly to each other in the inhibition ELISA, *L. perenne* pollen showed more potent inhibition at lower concentrations than *P. pratense* pollen indicating allergenic differences that might be accounted for by differences between the group 5 allergens of

Pooideae grass pollens [24, 36]. IgE reactivity levels, titres and inhibition assays with the purified group 1 allergens were consistent with the observations made for whole pollen extracts, indicating that the group 1 grass pollen allergens make a major contribution to the total serum IgE reactivity. Notably, IgE reactivity of serum 4 with Lol p 1 was not completely inhibited by Pas n 1, indicating antigenic differences between the allergens in addition to shared epitopes. Additional epitopes unique to Pas n 1, Cyn d 1 or Lol p 1 are likely to contribute to polyclonal serum IgE reactivity with these allergens.

Basophil activation tests are sensitive *in vitro* assays closely resembling the *in vivo* symptoms elicited in response to allergen challenge [40, 41]. The results of this study show similar maximal basophil activation levels to

both subtropical and temperate grass pollens in patients from temperate climate regions. However, basophil sensitivity is significantly lower to the subtropical grasses, which correlates with the IgE inhibition data supporting the theory of higher IgE reactivity to temperate climate grass pollen allergens in these patients from a temperate region. Notably, *in vitro* basophil activation by *P. notatum* pollen provides strong evidence for the likely ability of this subtropical grass to exacerbate symptoms in patients from a temperate climate through cross-reactivity with shared epitopes present in orthologous allergens. Given the extended pollination season of *P. notatum* [26], this cross-sensitivity is likely to be clinically significant for patients in temperate regions where pollination of temperate grasses is characterized by a narrow spring peak.

Although this study focused mainly on patients with grass pollen allergy from a temperate region, we also present for the first time data on IgE reactivity of a panel of patients living in a subtropical region. In contrast to patients from a temperate region, subjects from a subtropical region showed similar IgE reactivity with *P. notatum* and *L. perenne* pollen. For these patients, reciprocal IgE cross-inhibition was observed between *L. perenne* and *P. notatum* pollen but there appeared to be a stronger avidity interaction with pollen of subtropical compared with temperate grasses.

Our findings of both shared and distinct immunological reactivity of *P. notatum* and *L. perenne* pollen, including their major group 1 allergens, suggest that efficacy of immunotherapy for grass pollen allergy could be increased by inclusion of *P. notatum* in the grass pollen immunotherapy preparations, especially for those patients primarily sensitized to *P. notatum* grass pollen. The efficacy of immunotherapy for *P. notatum* pollen allergy by current immunotherapy treatments that lack *P. notatum* pollen has yet to be fully investigated. Phillips *et al.* [42] showed a lack of *in vivo* cross-reactivity between

L. perenne and *P. notatum* pollens in a small number of patients by nasal challenge. An early study by Leveangood and colleagues showed that immunotherapy in 10 grass pollen-allergic patients with a mixture of extracts of *P. pratense* pollen and *C. dactylon* pollen reduced the skin prick weal size for most grasses including *P. notatum* pollen but the number of patients was small and SPT is not reliably quantitative [43].

In conclusion, our observations indicate that *P. notatum* pollen is an important allergen in both temperate and subtropical regions, with clinically relevant cross-reactivity with temperate grasses. However, the lack of reciprocal cross-inhibition and incomplete inhibition of IgE reactivity in sera of patients from a temperate region indicate species-specific recognition of subtropical and temperate grass pollens. Further research is required to quantify the contribution of subtropical grass pollens to the burden of AR and asthma in populations from subtropical environments where these grasses predominate. The desirability for representation of subtropical grass pollen allergens in immunotherapy vaccine preparations for patients primarily sensitized with subtropical grass pollens should be evaluated.

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