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Unique and Cross-Reactive T Cell Epitope Peptides of the Major Bahia Grass Pollen Allergen, Pas n 1

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Key Words
T cell epitopes · T cell cross-reactivity · Bahia grass pollen allergy · Allergic rhinitis

Abstract
Background: Bahia grass pollen (BaGP) is a major cause of allergic rhinitis. Subcutaneous allergen-specific immunotherapy is effective for grass pollen allergy, but is unsuitable for patients with moderate to severe asthma due to the risk of anaphylaxis. T cell-reactive but IgE nonreactive peptides provide a safer treatment option. This study aimed to identify and characterize dominant $CD4^+$ T cell epitope peptides of the major BaGP allergen, Pas n 1. ***Methods:*** Pas n 1-specific T cell lines generated from the peripheral blood of BaGP-allergic subjects were tested for proliferative and cytokine response to overlapping 20-mer Pas n 1 peptides. Cross-reactivity to homologous peptides from Lol p 1 and Cyn d 1 of Ryegrass and Bermuda grass pollen, respectively, was assessed using Pas n 1 peptide-specific T cell clones. MHC class II restriction of Pas n 1 peptide T cell recognition was determined by HLA blocking assays and peptide IgE reactivity tested by dot blotting. ***Results:*** Three Pas n 1 peptides showed dominant T cell reactivity; 15 of 18 (83%) patients responded to one or more of these peptides. T cell clones specific for dominant Pas n 1 peptides showed evidence of species-specific T cell reactivity as well as cross-reactivity with other group 1 grass pollen allergens. The dominant Pas n 1 T cell epitope peptides showed HLA binding diversity and were non-IgE reactive. ***Conclusions:*** The immunodominant T cell-reactive Pas n 1 peptides are candidates for safe immunotherapy for individuals, including those with asthma, who are allergic to Bahia and possibly other grass pollens.
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Introduction

Grass pollen allergens are a major cause of seasonal allergic rhinitis and asthma which are rapidly increasing health problems, particularly in developed countries [1]. Much research has focused on temperate grasses, but subtropical grasses such as *Paspalum notatum* (Bahia grass) are also significant sources of grass pollen allergens. Native to Central and South America, Bahia grass is now also grown widely in lawns, feed crops and orchard underplanting in temperate as well as subtropical regions of North America, Australia, Africa and Asia [2]. *P. notatum* has a protracted period of pollination

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extending to late summer [3]. We reported previously that Pas n 1 is the most important allergen of *P. notatum,* showing serum IgE reactivity for 85% grass pollen-allergic patients in a temperate zone [4]. Unlike other Pooideae grasses, *P. notatum* does not have group 2 or 5 allergens. Consistent with the 64–66% amino acid identity between Pas n 1 and the group 1 allergens of temperate grasses, we showed clinically relevant IgE cross-reactivity between *P. notatum* and *Lolium perenne* (Ryegrass) but also evidence for Pas n 1 species-specific IgE reactivity [5]. Others have shown unique IgE reactivity with Bahia grass pollen (BaGP) allergens that was not blocked by other grass pollens [6]. Given the phylogenetic and immunological differences between BaGP allergens and those of other grass pollens and the increasing distribution of Bahia grass, specific treatment of grass pollen allergy should also target reactivity to BaGP allergens.

Specific immunotherapy (SIT) has been successfully used to treat grass pollen allergy [7] and has been shown to decrease the risk of asthma development [8, 9]. However, conventional grass pollen SIT uses unmodified complex allergen extract mixtures that are difficult to standardize [10] and subcutaneous SIT with these extracts is unsuitable for patients with moderate-to-severe asthma due to the risk of anaphylaxis [11]. The efficacy of SIT is associated with the alteration of T cell responses to allergens including the induction of T regulatory cells (Treg) [12, 13]), anergy [14] and immune deviation from predominantly Th2 to Th1/Th0 type [15, 16]. Targeting allergen-specific $CD4^+$ T cells using T cell epitope-based peptides offers an alternative and safer approach for SIT [17, 18]. Unlike native allergens, short T cell-epitope-based allergen peptides do not activate basophils and mast cells to release inflammatory mediators, as they fail to cross-link cell-bound allergen-specific IgE. Peptides can also be produced to high pharmaceutical standards, with improved purity, consistency and composition. T cell epitope-based peptides from the major cat allergen Fel d 1 have recently shown safety and efficacy in clinical trials for cat allergy [19].

The objective of our study was to characterize T cell reactive sites of the major BaGP allergen, Pas n 1, in order to identify therapeutic candidates for peptide SIT. T cell responses of BaGP-allergic subjects to the group 1 allergens of Ryegrass and Bermuda grass were also investigated. The latter grasses represent separate subfamilies of clinically important grasses (Pooideae and Chloridoideae, respectively) from Bahia grass (Panicoideae). All three grasses are abundant sources of pollen allergens in temperate and subtropical regions. These data will aid in the development of more refined immunotherapy for allergic disease induced by Bahia and possibly other grasses.

Materials and Methods

Subjects

BaGP-allergic individuals were recruited from the Alfred Hospital Allergy Clinic, Melbourne, Australia. Donors were selected on the basis of clinical history, positive BaGP-specific IgE [ImmunoCAP (Phadia Pty Ltd, Uppsala, Sweden) class ≥3] and/or positive skin-prick test (SPT; wheal diameter ≥5 mm). Patient demographics are shown in table 1. Selected subjects were HLA-typed (HLA-DRB1, HLA-DQB1 and HLA-DPB1, exon 2) by the Victorian Transplantation and Immunogenetics Service, Melbourne, Australia. The study was approved by the Alfred Hospital ethics committee, and informed written consent was obtained from each subject.

Antigens

Unfractionated aqueous BaGP extract was prepared as described previously [20]. Pas n 1 was purified from the extract by ammonium sulphate precipitation, hydrophobic interaction chromatography and size exclusion chromatography as described [21].

Peptides were purchased from Mimotopes (Clayton, Australia) and reconstituted at 2 mg/ml in 10% dimethyl sulfoxide/PBS. These comprised peptides spanning the entire Pas n 1 sequence ([4]; Genbank accession number EU327342; 20-mers, 11 amino acid overlap except for the last peptide which had a 15 amino acid overlap), as well as 20-mer peptides of Lol p 1 and Cyn d 1 homologous with identified dominant T cell-reactive Pas n 1 peptides [22, 23]. Antigens were confirmed as nonmitogenic and nontoxic as described [23].

IgE Immunoblotting

BaGP extract proteins were separated by 14% sodium dodecylsulphate polyacrylamide gel electrophoresis using mini-gels (SDS-PAGE; Invitrogen, Carlsbad, Calif., USA), transferred to nitrocellulose membranes, incubated with BaGP-allergic subject sera (diluted 1:20) and bound-IgE detected as described [20].

Dot-Blot IgE Binding Assay

Nitrocellulose membrane (0.2 μm pore size) was dotted with 10 μg of Pas n 1 protein or 20 μg of Pas n 1 peptide, blocked with TRIS-buffered saline containing 0.05% Tween 20 and 5% sucrose, incubated with individual subject sera (diluted 1:10) and IgE binding was assessed as described [24].

Peripheral Blood Mononuclear Cell Proliferative Response to BaGP and Pas n 1

Peripheral blood mononuclear cells (PBMC; 1×10^5 cells/well) were cultured in triplicate in 96-U-well plates in 200 μl complete RPMI medium [cRPMI; RPMI 1640 medium (Gibco BRL, N.Y., USA) supplemented with 2 mmol/l GlutaMAX I (Gibco)], 0.05 mg/ml gentamicin, 100 IU/ml penicillin-streptomycin and 5% heat-inactivated human AB serum (Sigma Aldrich, Mo., USA)] for 7 days in the presence of BaGP (25, 50 and 100 μg/ml), Pas n 1 (5, 10 and 20 μg/ml) or medium alone. Proliferation was

Table 1. Clinical characteristics of BaGP-allergic subjects

Subject	Age (years)	Sex	Clinical diagnosis	SPT (mean diameter, mm)			Serum-specific IgE (kU_A/l)			
				BaGP	RGP	BeGP	Total IgE	BaGP	RGP	BeGP
1	21	Male	AR	9	12	8	274	53	>100	6.86
2	34	Female	A,AR	7	6	10	119	13.7	18	5.05
3	23	Female	A,AR	9	10	12	646	93.4	>100	64.9
4	25	Female	AR	11	3	10	1,113	>100	>100	53.7
5	25	Male	AR	9	7	10	158	41.6	59.6	23.9
6	32	Male	AR	10	13	10	594	17.8	>100	9.36
7	34	Male	AR	13	7	6	119	14	41.5	9.26
8	25	Male	AR	15	10	7	701	22.3	79.8	7.4
9	42	Female	AR	8	10	8	151	4.95	14.9	2.16
10	38	Female	A,AR	9	14	10	1,245	92.5	>100	44.1
11	28	Female	AR	8	8	12	867	>100	>100	>100
12	21	Male	AR	6	16	9	86	4.48	11	2.27
13	30	Female	A,AR	10	7	7	288	51.6	>100	16.4
14	44	Male	AR	7	5	4	369	9.38	20.6	3.37
15	40	Male	A,AR	10	7	6	496	17.6	8.52	13
16	34	Female	A,AR	6	3	5	132	19	37.7	17.6
17	63	Male	AR	5	9	5	136	6.21	8.3	0.9
18	52	Male	AR	12	6	12	1,222	39.7	38	9.79
Median				9.0	7.5	8.5	328.5	20.7	50.55	9.75[a]
Interquartile range				7.0–10.3	6.0–10.5	6.0–10.0	135–742	12.6–62.9	17.2–100	4.6–29.0

A = Asthma; AR = allergic rhinitis.

[a] Serum-specific IgE reactivity with BeGP was significantly lower ($p < 0.001$) than with RGP (Friedman's ANOVA with Dunn's multiple comparison test for nonparametric data).

measured by the incorporation of tritiated thymidine (^{3}H-TdR; 0.5 μCi/well) during the last 16 h of culture. Stimulation indices (SI) were calculated as the counts per minute (cpm) for cultures with allergen divided by the cpm for cultures without stimulus, and SI values ≥2.5 were considered positive.

Generation of Short-Term Oligoclonal Pas n 1-Specific TCL

Pas n 1-specific oligoclonal T cell lines (TCL) were generated by culturing PBMC (2.5×10^6 cells/well) in 24-well plates (Costar, Mass., USA) with Pas n 1 (30 μg/ml) in cRPMI at 37°C in a 5% CO_2 humidified incubator. At day 7, cells were washed and restimulated in 24-well plates (5×10^5 cells/well) with Pas n 1 (30 μg/ml) in the presence of irradiated (3,000 rads) autologous PBMC (5×10^5 cells/well) as a source of antigen-presenting cells (APC). At day 2 following restimulation, recombinant (r) IL-2 (20 U/ml; Cetus, Emeryville, Calif., USA) was added. At day 4 after restimulation, 1 ml of culture medium was removed and replaced with fresh cRPMI and rIL-2 (20 U/ml). For certain experiments to increase cell numbers, TCL were restimulated as described above for a further week. In all experiments, T cells were rested for 7 days after the last addition of antigen and APC before testing for their reactivity to peptides.

Generation of Pas n 1 Peptide-Specific T Cell Clones

Pas n 1 peptide-specific T cell clones (TCC) were generated from PBMC of subjects with BaGP allergy using 5,6 carboxyfluorescein diacetate succinimidylester (CFSE)-based methodology [25]. For this, CFSE-stained PBMC (2×10^5 cells/well, 150 μl) were cultured in 96-U-well plates with Pas n 1 peptides (10 μg/ml) or as controls, tetanus toxoid (TT; 10 LfU/ml; Statens Serum Institute, Copenhagen, Denmark) or cRPMI alone, for 7 days at 37°C. After staining with phycoerythrin-labelled anti-CD4 and 7AAD (BD Pharmingen, San Diego, Calif., USA), $CD4^+CFSE^{dim}7AAD^-$-proliferating antigen-specific T cells were sorted (1 cell/well) into 96-U-well plates containing irradiated feeder cells (1×10^5 PBMC from each of 2 unrelated donors and 2×10^4 EBV-transformed JY cells), anti-CD3 (clone OKT-3; 30 ng/ml), rIL-2 (10 U/ml; Cetus) and Fungizone (Invitrogen). Cells were cultured for 10–14 days, feeding with IL-2 as required, then transferred to 48-well plates and tested for peptide specificity as described [25]. Pas n 1 peptide-specific TCC and control TT-specific TCC were expanded with irradiated feeders (12×10^6 PBMC from each of 2 donors and 2.4×10^6 EBV-transformed JY cells in 8 ml), anti-CD3 (30 ng/ml) and rIL-2 (50 U/ml) in T25 culture flasks (BD, Franklin Lakes, N.J., USA) for 10–12 days.

T Cell Proliferation Assays

Proliferation assays were performed on 72-hour duplicate or triplicate cultures of TCL or TCC in 96-U-well plates containing 1×10^4 T cells/well and 1×10^4 irradiated (5,000 rad) autologous EBV-transformed PBMC (EBV-B cells) as APC together with antigens as specified or as a negative control, cRPMI alone. Cells

were pulsed with ^{3}H-thymidine (0.5 μCi/well) for the last 16 h, uptake recorded as mean cpm of replicate cultures and SI determined as before.

T Cell Receptor Vβ Analysis

To assess the clonality of Pas n 1 peptide-specific TCC, especially those demonstrating cross-reactivity with homologous peptides of RGP and BeGP, the T cell receptor Vβ repertoire was determined using the IOTest BetaMark TCR Vbeta Repertoire Kit as per manufacturer's instructions (Beckman Coulter, Krefeld, Germany) with the addition of APC-conjugated anti-CD4 antibody (BD Pharmingen) for T cell identification and 7AAD (BD Pharmingen) for the exclusion of dead cells.

HLA Class II Blocking Assays

TCC and irradiated autologous EBV-B cells (1×10^4 of each) were incubated with 0.1–10 μg/ml blocking monoclonal antibodies (mAb) against HLA-DR (L243, BD Pharmingen), HLA-DQ (SVP-L3), HLA-DP (B7/21) (gifts from S. Mannering, St Vincent's Research Institute, Melbourne, Australia) or isotype control antibodies (IgG2a, BD Pharmingen; IgG_1, BioLegend, San Diego, Calif., USA) for 1 h at 37°C before adding peptides (10 μg/ml) and testing the proliferative response as above.

Cytokine Detection in Culture Supernatants

Levels of IL-4, IL-5, IL-10, IFN-γ and TNF-α cytokines in the supernatants of TCC proliferation assay cultures were measured using the Cytometric Bead Array Human Cytokines kit (BD Biosciences, San Jose, Calif., USA) according to the manufacturer's instructions. The standard curve ranged from 0 to 5,000 pg/ml and the limits of detection were 2.6, 2.4, 2.8, 7.1 and 2.8 pg/ml for IL-4, IL-5, IL-10, IFN-γ and TNF-α, respectively.

Results

Demographics of Bahia Grass Pollen-Allergic Subjects

Eighteen BaGP-allergic subjects were studied (table 1). All subjects also showed positive specific IgE to Ryegrass pollen (RGP) and Bermuda grass pollen (BeGP) by SPT and/or ImmunoCAP. Allergic reactivity by SPT and ImmunoCAP was comparable for BaGP and RGP, whereas subjects showed significantly lower ImmunoCAP reactivity with BeGP than with RGP. All 14 subjects for whom additional serum was available for this study showed IgE reactivity to Pas n 1 by immunoblotting (data not shown).

Primary T Cell Response to BaGP and Pas n 1 Allergens

PBMC from all 18 BaGP-allergic subjects were screened for reactivity to BaGP extract and purified Pas n 1 in 7-day proliferation assays to determine the optimal antigen concentration for the generation of short-term Pas n 1-specific TCL. All subjects showed strong proliferative responses to BaGP extract with a median maximal SI of 13.4 [interquartile range (IQR) 10.2–24.1]. Pas n 1 also induced positive proliferative responses in all but one (subject 4) of the BaGP-allergic subjects tested, with a median maximal SI of 9.6 (IQR 4.0–20.1). The primary proliferative response to Pas n 1 was typically dose-dependent and 30 μg/ml was selected as the optimal concentration for generation of Pas n 1-specific TCL.

Identification of Pas n 1 Peptides Containing Dominant and Subdominant $CD4^+$ T Cell Epitopes

Oligoclonal Pas n 1-specific TCL were generated from the PBMC of each of the 18 BaGP-allergic patients. To identify the highly T cell-reactive regions of Pas n 1, proliferative responses of the TCL to 26 individual overlapping Pas n 1 20-mer peptides spanning the entire Pas n 1 sequence were determined (table 2). Each of the 18 Pas n 1-specific TCL responded to at least one peptide. Twelve (67%) Pas n 1-specific TCL responded to more than one Pas n 1 peptide. Pas n 1 (100–119), (154–173) and (217–236) were the most frequently and strongly recognized peptides with 7 (39%), 8 (44%) and 6 (33%) of the TCL reacting to these peptides, respectively. The three dominant peptides encompass distinct regions of Pas n 1 indicating that each peptide contains at least one separate and discrete T cell epitope. One or more of the dominant Pas n 1 peptides was recognized by TCL from 15 (83%) of the 18 BaGP allergic subjects, with TCL from 2 (11%) subjects recognizing all three dominant peptides.

Pas n 1 (127–146) and (190–209) were the next most frequently recognized (subdominant) peptides. Both of these subdominant peptides induced a response in Pas n 1-specific TCL from 5 (27.8%) subjects. Combined with the three dominant peptides, these 5 peptides induced T cell reactivity in 17 (94.4%) of the 18 BaGP-allergic subjects.

Characterization of Cross-Reactivity of Pas n 1-Specific T Cells with Group 1 Grass Pollen Allergens, Lol p 1 and Cyn d 1

The complete sequence of Pas n 1 has 64 and 66% amino acid identity with the group 1 grass pollen allergens Lol p 1 of RGP and Cyn d 1 of BeGP, respectively [4]. Table 3 shows the sequence comparisons between the dominant T cell-reactive peptides of Pas n 1 and homologous peptides of Lol p 1 and Cyn d 1. The amino acid identity and similarity with the homologs is highest for Pas n 1 (100–119) and least for Pas n 1 (217–236). Pas n 1-specific TCL from BaGP-allergic subjects that reacted to the dominant Pas n 1 peptides were therefore assessed for reactivity to the homologous peptides of Lol p 1 and Cyn d 1

Table 2. TCL proliferative responses to 20-mer peptides spanning Pas n 1

Subjects	Pas n 1	Pas n 1 (1–20)	Pas n 1 (10–29)	Pas n 1 (19–38)	Pas n 1 (28–47)	Pas n 1 (37–56)	Pas n 1 (46–65)	Pas n 1 (55–74)	Pas n 1 (64–83)	Pas n 1 (73–92)	Pas n 1 (82–101)	Pas n 1 (91–110)	Pas n 1 (100–119)	Pas n 1 (109–128)	Pas n 1 (118–137)	Pas n 1 (127–146)	Pas n 1 (136–155)	Pas n 1 (145–164)	Pas n 1 (154–173)	Pas n 1 (163–182)	Pas n 1 (172–191)	Pas n 1 (181–200)	Pas n 1 (190–209)	Pas n 1 (199–218)	Pas n 1 (208–227)	Pas n 1 (217–236)	Pas n 1 (222–241)
1	NT																										
2																											
3																											
4																											
5																											
6																											
7																											
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10																											
11																											
12																											
13																											
14																											
15																											
16																											
17																											
18																											
													38.9%						44.4%							33.3%	
																			83.3%								

Background cpm with medium alone ranged from 136–13,993 cpm. Most frequently recognized peptides (boxed). NT = not tested. White boxes: SI <2.5. Gray boxes: SI ≥2.5. Black boxes: SI ≥5.

(fig. 1). Two TCL specific for each dominant peptide were tested. Proliferation was observed for Pas n 1 (100–119)-specific and Pas n 1 (154–173)-specific TCL in response to homologous peptides of Lol p 1 and Cyn d 1 (fig. 1a–d). However, no proliferation was observed to homologous peptides from Lol p 1 and Cyn d 1 for the Pas n 1 (217–236)-specific TCL (fig. 1e, f).

To confirm whether the multiple allergen reactivity observed in some Pas n 1-specific TCL was due to cross-reactivity of a clonal T cell population, Pas n 1 peptide-specific TCC were generated and tested for proliferation to homologous peptides from Lol p 1 and Cyn d 1 (fig. 2). Eight (50%) of the 16 Pas n 1 (100–119)-specific TCC did not react to either of the homologous peptides from Lol p 1 or Cyn d 1, while 4 (25%) cross-reacted to one of the homologous peptides and 4 (25%) cross-reacted to both Lol p 1 and Cyn d 1 peptides. Three (37.5%) of the 8 Pas n 1 (154–173)-specific TCC did not react with either of the homologous peptides from Lol p 1 or Cyn d 1. Two (25%) Pas n 1 (154–173)-specific TCC cross-reacted to 1

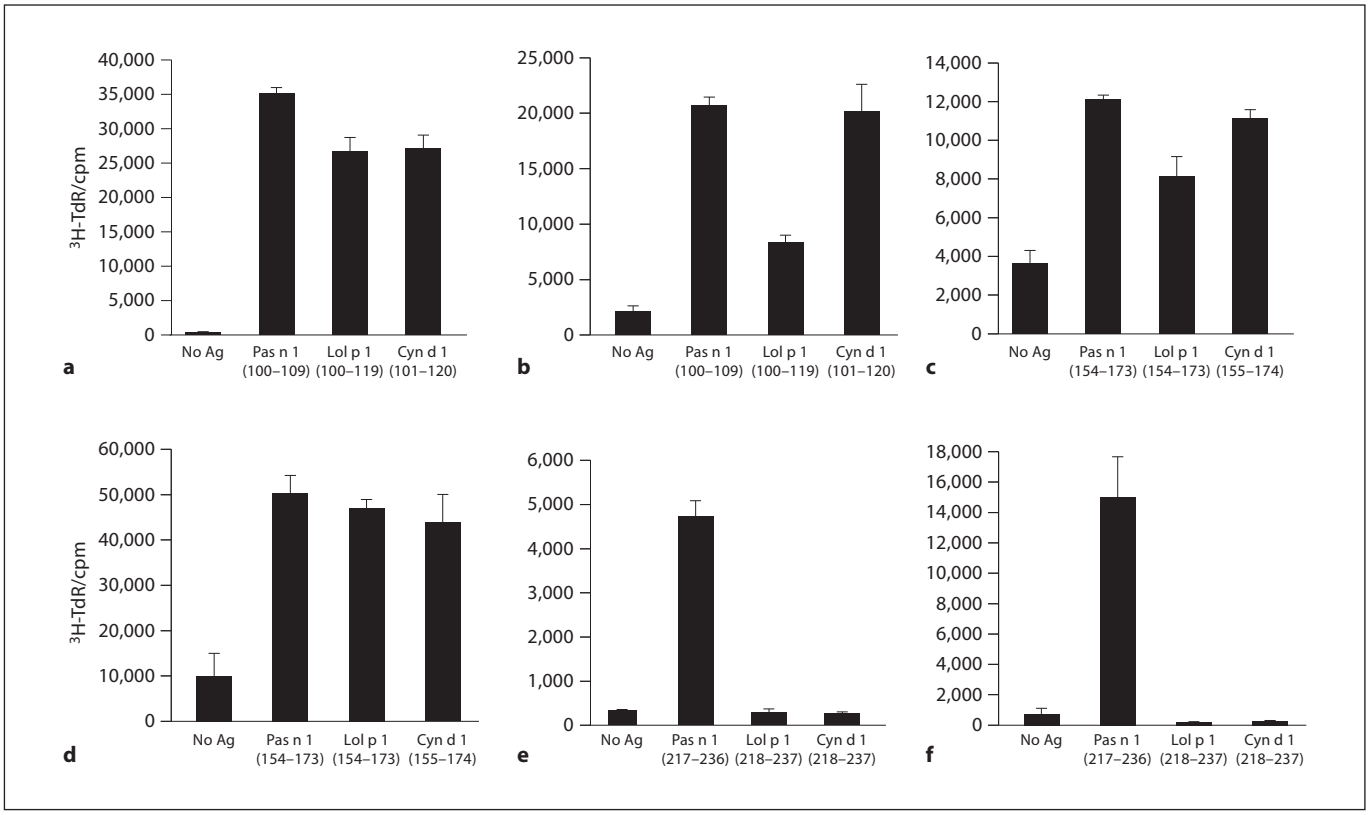

Fig. 1. Pas n 1 dominant peptide-specific TCL proliferative response to homologous peptides from Lol p 1 and Cyn d 1. Two TCL specific for each of the 3 dominant peptides were tested. Pas n 1 (100–119)-specific TCL from patients 13 (**a**) and 12 (**b**), Pas n 1 (154–173)-specific TCL from patients 5 (**c**) and 2 (**d**) and Pas n 1 (217–236)-specific TCL from patients 13 (**e**) and 15 (**f**). Mean cpm of triplicate cultures + SD shown. Ag = Antigen; ^{3}H-TdR = ^{3}H-thymidine.

Table 3. Sequence alignments of Pas n 1 (100–119), Pas n 1 (154–173) and Pas n 1 (217–236) with homologous Lol p 1 and Cyn d 1 peptides[a]

Peptide	Amino acid sequence	Identity	Similarity[b]
Pas n 1 (100–119)	IAPYHFDLSGKAFGAMAKPG	–	–
Lol p 1 (100–119)	**********H***S***K*	85%	95%
Cyn d 1 (101–120)	**A**********K*	90%	90%
Pas n 1 (154–173)	GSNPNYLAMLVKFVADDGDI	–	–
Lol p 1 (154–173)	A*******I***Y*DG***V	70%	85%
Cyn d 1 (155–174)	**S*****L***YA*G**N*	70%	85%
Pas n 1 (217–236)	GKKVIAQDVIPVNWKPDTVY	–	–
Lol p 1 (218–237)	*T*SEFE****EG**A**S*	55%	60%
Cyn d 1 (218–237)	*GH*EQD****ED*******	65%	80%

* Identical amino acids. **Bold**: conservative exchanges of amino acids. Underlined: semi-conservative exchanges of amino acids.

[a] Lol p 1.0101 isoform (mature protein) and Cyn d 1.0102 isoform.

[b] Peptide sequences were aligned with ClustalW using the Blosum protein comparison matrix. The percentage identity was calculated by the number of identities between the two sequences divided by the length of the alignment. The percentage similarity was based on the proportion of identical and conservative amino acid substitutions between sequences.

Pas n 1 (100–119)-specific TCC

		Pas n 1 (100–119)	Lol p 1 (100–119)	Cyn d 1 (101–120)
Subject 13	TCC1	2.8		
Subject 12	TCC 6	13.6		14.1
	TCC 1	5.0		
	TCC 11	11.2		
	TCC 14	7.7		
Subject 15	TCC 10	76.9	45.2	80.9
	TCC 39	62.9	79.3	69.4
	TCC 23	4.6	3.7	6.4
	TCC 30	14.3	16.6	16.5
	TCC 34	132.0		103.4
	TCC 3	23.3		19.6
	TCC 11	74.3		97.8
	TCC 25	186.4		
	TCC 13	42.0		
	TCC 14	11.8		
a	TCC 6	40.9		

Pas n 1 (154–173)-specific TCC

		Pas n 1 (154–173)	Lol p 1 (154–173)	Cyn d 1 (155–174)
Subject 15	TCC 18	27.7	38.9	29.8
	TCC 20	61.9	50.4	
	TCC 17	37.6		
Subject 8	TCC 18	5.6	7.5	7.0
Subject 10	TCC 2.1	4.0	4.3	4.9
	TCC 32	25.0	25.6	
	TCC 2	36.8		
b	TCC 27	30.4		

Pas n 1 (217–236)-specific TCC

		Pas n 1 (217–236)	Lol p 1 (218–237)	Cyn d 1 (218–237)
Subject 10	TCC 9	12.5		9.0
	TCC 2	52.3		
	TCC 12	71.9		
Subject 13	TCC 3	2.5		
	TCC 4	16.9		
	TCC 5	45.1		
	TCC 10	65 .1		
	TCC 12	128.3		
Subject 15	TCC 25	10.8		
	TCC 29	7.3		
	TCC 32	20.6		
c	TCC 37	16.3		

Fig. 2. Pas n 1-dominant peptide-specific TCC proliferative responses to homologous peptides from Lol p 1 and Cyn d 1. Summary of reactivity of all the Pas n 1 peptide-specific TCC generated. SI <2.5 (white boxes), 5> SI ≥2.5 (gray boxes) and SI ≥5 (black boxes).

homologous peptide and 3 (37.5%) cross-reacted to both homologous peptides. However, all except 1 of the 12 (91.7%) Pas n 1 (217–236)-specific TCC did not cross-react to either of the homologous peptides from Lol p 1 or Cyn d 1. Only one of 12 Pas n 1 (217–236)-specific TCC cross-reacted to the equivalent Cyn d 1 peptide. Clonality of TCC specific for each dominant Pas n 1 peptide was supported by T cell receptor Vβ analysis. In particular, TCC demonstrating cross-reactivity with homologous RGP and BeGP peptides expressed a single Vβ chain type.

Cytokine Profile of Pas n 1 Peptide-Specific TCC

The levels of cytokines (IL-4, IL-5, TNF, IFN- γ and IL-10) were tested in the culture supernatants of Pas n 1 peptide-specific TCC following stimulation with Pas n 1 peptide (10 μg/ml) or homologous peptides from Lol p 1 and Cyn d 1 (table 4) to further confirm reactivity of T cells to the dominant peptides and their homologs. The cytokine profiles varied, with some allergen-stimulated TCC producing predominantly Th2-type cytokines IL-4 and IL-5, and others producing high levels of IFN-γ and TNF and/or the regulatory cytokine, IL-10. In general, levels of cytokines produced by the TCC mirrored the proliferative responses to peptides. Where TCC showed reactivity to 1 or more homologous peptides, cytokine profiles were comparable to those for the 'parent' Pas n 1 peptide.

HLA Restriction Specificity of Dominant Pas n 1 Peptide T Cell Epitopes

HLA class II typing was performed on 8 BaGP-allergic subjects reactive to the dominant Pas n 1 T cell epitope peptides (table 5). To determine the HLA class II restriction of each peptide T cell epitope, anti-HLA-DR, anti-HLA-DP and anti-HLA-DQ mAb were used to block epitope presentation to T cells. For each TCC tested, epitope recognition was inhibited by 1 or more HLA mAb in a dose-dependent manner. Isotype control antibodies (10 μg/ml) had no effect (data not shown). HLA-DR restriction was observed for 5 Pas n 1 (100–119)-specific TCC from 3 subjects indicating preferential presentation by HLA-DR. To assess the potential for degeneracy of epitope presentation by allelic variants of HLA-DR, HLA alleles of the 5 subjects recognizing Pas n 1 (100–119) were compared (table 5). The absence of a

Table 4. Summary of cytokine production by 10 Pas n 1 peptide-specific TCC from 5 donors

	Cytokine production (pg/ml)				
	IL-4	IL-5	TNF	IFN-γ	IL-10
Subject 13 Pas n 1 (100–119) TCC1					
No antigen	3.88	5.04	57.62	95.54	501.38
Pas n 1 (100–119)	26.95	54.88	50.88	861.41	496.89
Lol p 1 (100–119)	3.88	5.04	47.81	117.15	621.56
Cyn d 1 (101–120)	2.6	3.54	44.44	90.94	441.9
Subject 12 Pas n 1 (100–119) TCC6					
No antigen	0	1.08	6.83	0	29.83
Pas n 1 (100–119)	6,243.4	7,717.1	828.55	1,393.8	1,010
Lol p 1 (100–119)	0	1.05	3.5	0	25.59
Cyn d 1 (101–1,120)	3,618.6	6,588.8	347.25	565.71	259.76
Subject 12 Pas n 1 (100–119) TCC14					
No antigen	0	2.69	11.13	4.99	25.88
Pas n 1 (100–119)	9.25	173.14	435.2	3,682.1	60.92
Lol p 1 (100–119)	0	3.54	9.31	9.15	25.29
Cyn d 1 (101–120)	0	2.61	7.94	3.92	27.4
Subject 15 Pas n 1 (154–173) TCC20					
No antigen	0	2.33	21.68	2.05	1.58
Pas n 1 (154–173)	0	1.85	181.06	15.36	2.59
Lol p 1 (154–173)	0	5.41	43.34	1.95	1.33
Cyn d 1 (155–174)	0	4.37	12.4	2.29	0
Subject 15 Pas n 1 (154–173) TCC18					
No antigen	0	3.93	32.3	2.9	0
Pas n 1 (154–173)	3.71	10.59	59.74	3.58	0
Lol p 1 (154–173)	4.1	19.58	52.78	4.86	1.33
Cyn d 1 (155–174)	4.23	12.17	41.14	4.01	0

	Cytokine production (pg/ml)				
	IL-4	IL-5	TNF	IFN-γ	IL-10
Subject 8 Pas n 1 (154–173) TCC18					
No antigen	2.51	3.03	11.47	1.66	11.5
Pas n 1 (154–173)	1,561.8	4,254.1	2,444.6	1,236.2	44.61
Lol p 1 (154–173)	2,481.3	4,781.9	1,751.7	479.11	41.34
Cyn d 1 (155–174)	2,046.9	4,696.6	1,192.3	149.75	35.79
Subject 10 Pas n 1 (154–173) TCC32					
No antigen	45.39	126.49	9.73	13.61	22.63
Pas n 1 (154–173)	2,241.7	2,787.1	879.11	612.14	16.4
Lol p 1 (154–173)	3,593.8	3,958.6	953.63	166.85	18.71
Cyn d 1 (155–174)	3.34	73.99	5.19	22.37	19.7
Subject 10 Pas n 1 (217–236) TCC9					
No antigen	2.62	5.32	2.15	11.22	23.77
Pas n 1 (217–236)	1,577.4	5,375.1	242.89	69.32	297.79
Lol p 1 (218–237)	1.22	4.05	1.45	8.62	17.07
Cyn d 1 (218–237)	908.26	3,218.7	15.73	24.99	160.8
Subject 13 Pas n 1 (217–236) TCC5					
No antigen	0	0	5.19	1.52	17.1
Pas n 1 (217–236)	6.25	138.3	3,648.7	3,791.3	9.99
Lol p 1 (218–237)	0	0	4.17	1.62	18.75
Cyn d 1 (218–237)	0	0	1.65	1.49	18.27
Subject 15 Pas n 1 (217–236) TCC32					
No antigen	1.54	160.08	43.29	10.18	0
Pas n 1 (217–236)	5.48	184.18	129.58	13.17	1.7
Lol p 1 (218–237)	0	131.16	7.97	7.58	0
Cyn d 1 (218–237)	0	120.88	4.47	9.12	0

Gray highlights peptides to which TCC showed proliferation.

common HLA-DR allele between these subjects (the only overlaps were expression of HLA-DRB1*1101 by 2 subjects and HLA-DRB1*0401 by 2 others) indicates Pas n 1 (100–119) epitope presentation by at least 3 different HLA-DR molecules. HLA-DP and HLA-DR restriction was observed for 1 and 2 Pas n 1 (154–173)-specific TCC, respectively. Comparison of HLA class II alleles from all 5 subjects reactive to Pas n 1 (154–173) indicates epitope presentation by at least 3 HLA allelic variants. HLA-DR restriction was observed for 4 Pas n 1 (217–236)-specific TCC from 2 individuals. The absence of a common HLA-DR allele between all 4 subjects reactive to Pas n 1 (217–236) indicates epitope presentation by at least 4 HLA molecules.

Testing Candidate Peptides for Serum IgE Binding

Serum from 4 BaGP-allergic subjects (subjects 2, 10, 13 and 15) and 1 nonatopic control was tested for IgE binding by dot blot analysis (fig. 3). All patients showed clear IgE reactivity to Pas n 1, but none of the dominant T cell-reactive peptides bound patient serum IgE.

Discussion

Short T cell-reactive peptides of allergens provide a safe and effective alternative to conventional native allergen preparations for allergen-specific injection immunotherapy. In this study, we identify peptides suitable for developing peptide immunotherapy for Bahia grass pollen allergy. The most important IgE-reactive allergen of *P. notatum*, Pas n 1, was shown to also be a major T cell allergen of this grass pollen, and the specificity and phenotype of the human T cell response to Pas n 1 in BaGP-allergic subjects was characterized. By generating Pas n 1-specific TCL from a panel of BaGP-allergic subjects and

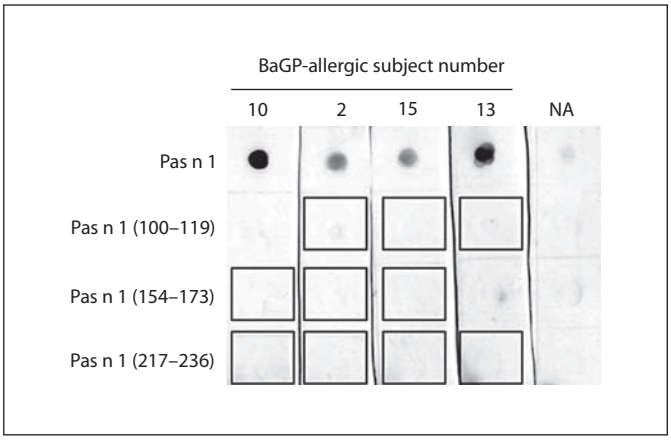

Fig. 3. IgE reactivity of dominant Pas n 1 peptides. Dot-blots showing binding of Pas n 1 or dominant Pas n 1 peptides by serum IgE from 4 BaGP-allergic subjects or as a control, a nonatopic (NA) donor. Boxes indicate Pas n 1 peptides to which the subject has T cell reactivity.

testing their reactivity against overlapping 20-mer peptides of Pas n 1, multiple T cell-reactive sites were identified throughout the Pas n 1 molecule, with 3 discrete regions showing dominance, Pas n 1 (100–119), Pas n 1 (154–173) and Pas n 1 (217–236). Together, the dominant peptides elicited responses in 83% of the BaGP-allergic subjects. HLA restriction of peptide presentation and the T cell cross-reactivity of these peptides with homologous peptides of the major group 1 allergens of RGP and BeGP were also examined. Our findings demonstrate that both species-specific and cross-reactive T cell epitopes may be recognized by allergen-specific T cells of grass pollen-allergic patients.

Dominant T cell-reactive peptides of Pas n 1 were identified on the basis of the frequency and strength of T cell reactivity in the panel of BaGP-allergic patients tested. In agreement with this selection is the high reactivity of dominant Pas n 1 peptide-specific T cells to the homologous peptides in the major group 1 grass pollen allergens of *L. perenne* (Lol p 1) and *Cynodon dactylon* (Cyn d 1). In our previous studies on RGP and BeGP, 2 of the 3 homologous peptides for both Lol p 1 and Cyn d were also found to be dominant T cell-reactive peptides [22–23]. Interestingly, 1 of the dominant T cell-reactive peptides, Pas n 1 (100–119), is located in a highly conserved region of the Pas n 1 molecule and contains a motif (HFD) that is conserved in >80 different sequences of the biologically important expansin family [26]. While addition of the subdominant T cell-reactive peptides to our domi-

Table 5. HLA class II typing of BaGP-allergic subjects T cell-reactive to dominant Pas n 1 peptides and HLA class II restriction specificity of dominant peptide presentation to TCC from these subjects

Subject	Pas n 1 peptide	DRB1	DRB3/4/5 and DPB1	DQB1 and DQA1	HLA class II restriction		
					Pas n 1 (100–119)	Pas n 1 (154–173)	Pas n 1 (217–236)
8	(154–173)	07:01, 11:01:01G	DPB1*02:01, 05:01:01G	DQB1*02:02, 03:01 and DQA1*02:01, 05:05/09	n.a.	n.d.	n.a.
10	(154–173), (217–236)	07:01, 12:02	DPB1*13:01:01G, 28:01	DQB1*02:02, 03:01 and DQA1*02:01, 06:01	n.a.	HLA-DR	HLA-DR
15	(100–119), (154–173), (217–236)	1101, 1401/54	DPB1*0101, 0402/0602	DQB1*0301/09/19/21/22, 0503	HLA-DR	HLA-DP	n.d.
13	(100–119), (217–236)	0401, 1404	DPB1*0401	DQB1*0301/09/19/21/22, 0503	HLA-DR	n.a.	HLA-DR
12	(100–119)	1101, 1301	DPB1*0401, 0901	DQB1*0301/09/19/21/22, 0603	HLA-DR	n.a.	n.a.
11	(100–119)	0301, 0401	DPB1*0401	DQB1*0201/02/04, 0301/09/19/21/22	n.d.	n.a.	n.a.
2	(100–119), (154–173), (217–236)	0403, 1104	DPB1*0201, 0402/0602	DQB1*0301/09/19/21/22, 0302	n.d.	n.d.	n.d.
5	(154–173)	0401, 0801	DPB1*0301/0502/2702, 0401	DQB1*0301/09/19/21/22,0402	n.a.	n.d.	n.a.

n.a. = Not applicable; n.d. = not determined.

nant peptide panel would increase the frequency of recognition by BaGP-allergic subjects, proof-of-concept studies on a peptide vaccine for cat allergy using dominant T cell-reactive Fel d 1 peptides demonstrate linked epitope suppression, indicating that the peptide mix for immunotherapy does not require all possible T cell epitopes in order to be effective [27]. Immunotherapy with single peptides representing immunodominant T cell epitopes can modulate the immune response to the complete allergen [27–28].

Importantly for use in therapy, immunodominant T cell epitope-based peptides should induce T cell reactivity in a genetically diverse population by binding to multiple HLA class II molecules. Our HLA blocking studies of Pas n 1 peptide-specific TCC indicate that Pas n 1 (100–119) and Pas n 1 (217–236) are HLA-DR restricted while Pas n 1 (154–173) can be presented by both HLA-DR and HLA-DP. Together with the HLA typing information for our subjects, these data demonstrate that each dominant peptide shows substantial HLA-binding degeneracy. Using the ProPred program (http://bic.uams.edu/mirror/propred/index.html) to predict MHC class II binding regions [29], Pas n 1 (100–119) would bind 13 of 51 (25%) HLA-DR molecules (including HLA-DRB1*1101 as expressed by subjects 12 and 15 of this study), Pas n 1 (154–173) would bind 32 of 51 (63%) HLA-DR molecules (including HLA-DRB1*0701 as expressed by subject 8) and Pas n 1 (217–236) would bind 27 of 51 (53%) HLA-DR molecules (including HLA-DRB1*0401 as expressed by subject 13). Interestingly, the subdominant Pas n 1 peptides are predicted to bind a higher proportion of HLA-DR molecules (75% for Pas n 1 (127–146) and 76% for Pas n 1 (190–209)) than the dominant Pas n 1 peptides, but it should be noted that the predicted MHC binding of a peptide does not necessarily correlate with T cell recognition. Such algorithms are useful for predicting potential T cell epitopes of allergens, but our experience shows that validation is required by testing clinical samples, as we have done in this study.

Although IgE cross-reactivity between different group 1 temperate grass pollen allergens has been demonstrated, the magnitude of response differs and both species-specific IgE epitopes and asymmetric patterns of IgE cross-reactivity are observed [30]. Cross-reactivity between Pas n 1 and other group 1 allergens of clinically important grass pollens at the T cell level has not been investigated before. Peptide binding of HLA class II molecules is promiscuous and a high degree of variability within an antigen is tolerable to retain functional T cell epitopes. Therefore, T cell cross-reactivity is an expected phenomenon for peptides with similar amino acid sequences [31]. In this study, we showed that Pas n 1-specific TCL from BaGP-allergic subjects reacted to homologous group 1 major allergens from Lol p 1 and Cyn d 1. In order to confirm cross-reactivity, Pas n 1 peptide-specific TCC were generated and screened for reactivity to corresponding peptides from Lol p 1 and Cyn d 1. We employed a highly specific approach to increase cloning efficiency by enriching for allergen-reactive T cells by in vitro allergen stimulation of CFSE-labelled PBMC and flow cytometric sorting of divided $CFSE^{low}$ $CD4^{+}$ T cells [32]. Cross-reactivity was observed in several Pas n 1 (100–119) and Pas n 1 (154–173) peptide-specific TCC, but in only one Pas n 1 (217–236)-specific TCC. These results are consistent with the higher degree of sequence identity between equivalent Pas n 1 (100–119) and Pas n 1 (154–173) peptides from Lol p 1 and Cyn d 1, compared to those for Pas n 1 (217–236). However, despite the high degree of homology, only 50% of Pas n 1 (100–119)-specific and 62.5% of Pas n 1 (154–173)-specific TCC cross-reacted with homologous peptides. The demonstration of cross-reactivity of Pas n 1 peptide-specific TCC suggests that the concurrent clinical reactivity to *P. notatum, L. perenne* and *C. dactylon* in BaGP-allergic subjects that we observed could be induced by T cell cross-reactivity. However, the different cross-reactivity patterns observed for Pas n 1 peptide-specific TCC in this study, and as reported previously for other grass pollen allergens [33–36], demonstrates that unique species-specific TCC also exist and that multiple T cell epitopes, unequally represented in related allergens, may be recognized by allergen-specific T cells of grass pollen-allergic patients.

Another important criterion for peptide selection for allergen immunotherapy is that the peptides do not bind and cross-link cell-bound IgE. This ensures that the risk of adverse events such as anaphylaxis during SIT is negligible compared with crude allergen extracts [37]. Our dot blots confirmed the lack of binding of serum IgE from BaGP-allergic subjects to either Pas n 1 (100–119), Pas n 1 (154–173) or Pas n 1 (217–236). Therefore, these dominant T cell epitopes of Pas n 1 are ideal candidates for peptide SIT of patients allergic to BaGP, including those with moderate to severe asthma. The cross-reactivity profiles identified in this study allow us to assess whether a single peptide from one grass pollen allergen would be effective for the treatment of patients allergic to homologous grass pollen allergens. Although a high degree of sequence homology and some cross-reactivity was observed in the Pas n 1 peptide-specific TCC, par-

ticularly for Pas n 1 (100–119) and (154–173), there was not complete coverage, which suggests the importance of using individual dominant peptides from each of the grasses for the most effective grass pollen allergy therapeutic.

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References

1 Asher MI, Montefort S, Bjorksten B, Lai CK, Strachan DP, Weiland SK, Williams H: Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC phases one and three repeat multicountry cross-sectional surveys. Lancet 2006;368: 733–743.

2 Clayton WD, Harman KT, Williamson H: Grassbase – the online world grass flora. http://wwwkeworg//data/grasses-dbhtml (last accessed Nov 2011).

3 Bass DJ, Delpech V, Beard J, Bass P, Walls RS: Late Summer and Fall (March–May) pollen allergy and respiratory disease in Northern New South Wales, Australia. Ann Allergy Asthma Immunol 2000;85:374–381.

4 Davies JM, Mittag D, Dang TD, Symons K, Voskamp A, Rolland JM, O'Hehir RE: Molecular cloning, expression and immunological characterisation of Pas n 1, the major allergen of Bahia grass *Paspalum notatum* pollen. Mol Immunol 2008;46:286–293.

5 Davies JM, Dang TD, Voskamp A, Drew AC, Biondo M, Phung M, Upham JW, Rolland JM, O'Hehir RE: Functional immunoglobulin E cross-reactivity between Pas n 1 of Bahia grass pollen and other group 1 grass pollen allergens. Clin Exp Allergy 2011;41:281–291.

6 Martin BG, Mansfield LE, Nelson HS: Cross-allergenicity among the grasses. Ann Allergy 1985;54:99–104.

7 Walker SM, Pajno GB, Lima MT, Wilson DR, Durham SR: Grass pollen immunotherapy for seasonal rhinitis and asthma: a randomized, controlled trial. J Allergy Clin Immunol 2001;107:87–93.

8 Jacobsen L, Niggemann B, Dreborg S, Ferdousi HA, Halken S, Host A, Koivikko A, Norberg LA, Valovirta E, Wahn U, Moller C: Specific immunotherapy has long-term preventive effect of seasonal and perennial asthma: 10-year follow-up on the PAT study. Allergy 2007;62:943–948.

9 Moller C, Dreborg S, Ferdousi HA, Halken S, Host A, Jacobsen L, Koivikko A, Koller DY, Niggemann B, Norberg LA, Urbanek R, Valovirta E, Wahn U: Pollen immunotherapy reduces the development of asthma in children with seasonal rhinoconjunctivitis (the PAT-study). J Allergy Clin Immunol 2002;109:251–256.

10 Jutel M, Cromwell O: Clinical results from vaccination with recombinant grass pollen allergens. Clin Dev Immunol 2006;13:389–394.

11 Bousquet J, Lockey R, Malling HJ, Alvarez-Cuesta E, Canonica GW, Chapman MD, Creticos PJ, Dayer JM, Durham SR, Demoly P, Goldstein RJ, Ishikawa T, Ito K, Kraft D, Lambert PH, Lowenstein H, Muller U, Norman PS, Reisman RE, Valenta R, Valovirta E, Yssel H: Allergen immunotherapy: therapeutic vaccines for allergic diseases. World Health Organization. Ann Allergy Asthma Immunol 1998;81:401–405.

12 Francis JN, Till SJ, Durham SR: Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy. J Allergy Clin Immunol 2003;111:1255–1261.

13 Gardner LM, Thien FC, Douglass JA, Rolland JM, O'Hehir RE: Induction of T 'regulatory' cells by standardized house dust mite immunotherapy: an increase in CD4+ CD25+ interleukin-10+ T cells expressing peripheral tissue trafficking markers. Clin Exp Allergy 2004;34:1209–1219.

14 Ebner C, Siemann U, Bohle B, Willheim M, Wiedermann U, Schenk S, Klotz F, Ebner H, Kraft D, Scheiner O: Immunological changes during specific immunotherapy of grass pollen allergy: reduced lymphoproliferative responses to allergen and shift from TH2 to TH1 in T-cell clones specific for Phl p 1, a major grass pollen allergen. Clin Exp Allergy 1997;27:1007–1015.

15 Bellinghausen I, Metz G, Enk AH, Christmann S, Knop J, Saloga J: Insect venom immunotherapy induces interleukin-10 production and a Th2-to-Th1 shift, and changes surface marker expression in venom-allergic subjects. Eur J Immunol 1997;27:1131–1139.

16 Jutel M, Akdis M, Blaser K, Akdis CA: Mechanisms of allergen specific immunotherapy – T-cell tolerance and more. Allergy 2006;61: 796–807.

17 Moldaver D, Larche M: Immunotherapy with peptides. Allergy 2011;66:784–791.

18 Rolland JM, Gardner LM, O'Hehir RE: Allergen-related approaches to immunotherapy. Pharmacol Ther 2009;121:273–284.

19 Worm M, Lee HH, Kleine-Tebbe J, Hafner RP, Laidler P, Healey D, Buhot C, Verhoef A, Maillere B, Kay AB, Larche M: Development and preliminary clinical evaluation of a peptide immunotherapy vaccine for cat allergy. J Allergy Clin Immunol 2011;127:89–97.

20 Davies JM, Bright ML, Rolland JM, O'Hehir RE: Bahia grass pollen specific IgE is common in seasonal rhinitis patients but has limited cross-reactivity with Ryegrass. Allergy 2005;60:251–255.

21 Drew AC, Davies JM, Dang TD, Rolland JM, O'Hehir RE: Purification of the major group 1 allergen from Bahia grass pollen, Pas n 1. Int Arch Allergy Immunol 154:295–298.

22 Burton MD, Papalia L, Eusebius NP, O'Hehir RE, Rolland JM: Characterization of the human T cell response to rye grass pollen allergens Lol p 1 and Lol p 5. Allergy 2002;57: 1136–1144.

23 Eusebius NP, Papalia L, Suphioglu C, McLellan SC, Varney M, Rolland JM, O'Hehir RE: Oligoclonal analysis of the atopic T cell response to the group 1 allergen of *Cynodon dactylon* (Bermuda grass) pollen: pre- and post-allergen-specific immunotherapy. Int Arch Allergy Immunol 2002;127:234–244.

24 Prickett SR, Voskamp AL, Dacumos-Hill A, Symons K, Rolland JM, O'Hehir RE: Ara h 2 peptides containing dominant CD4+ T-cell epitopes: candidates for a peanut allergy therapeutic. J Allergy Clin Immunol 2010; 127:608–615, e601–e605.

25 Mannering SI, Dromey JA, Morris JS, Thearle DJ, Jensen KP, Harrison LC: An efficient method for cloning human autoantigen-specific T cells. J Immunol Methods 2005;298: 83–92.

26 Yennawar NH, Li LC, Dudzinski DM, Tabuchi A, Cosgrove DJ: Crystal structure and activities of EXPB1 (Zea m 1), a beta-expansin and group-1 pollen allergen from maize. Proc Natl Acad Sci USA 2006;103:14664–14671.

27 Campbell JD, Buckland KF, McMillan SJ, Kearley J, Oldfield WL, Stern LJ, Gronlund H, van Hage M, Reynolds CJ, Boyton RJ, Cobbold SP, Kay AB, Altmann DM, Lloyd CM, Larche M: Peptide immunotherapy in allergic asthma generates IL-10-dependent immunological tolerance associated with linked epitope suppression. J Exp Med 2009; 206:1535–1547.

28 Hoyne GF, O'Hehir RE, Wraith DC, Thomas WR, Lamb JR: Inhibition of T cell and antibody responses to house dust mite allergen by inhalation of the dominant T cell epitope in naive and sensitized mice. J Exp Med 1993; 178:1783–1788.
29 Singh H, Raghava GP: ProPred: prediction of HLA-DR binding sites. Bioinformatics 2001; 17:1236–1237.
30 Esch RE, Klapper DG: Isolation and characterization of a major cross-reactive grass group I allergenic determinant. Mol Immunol 1989;26:557–561.
31 Boehncke WH, Loeliger C, Kuehnl P, Kalbacher H, Bohm BO, Gall H: Identification of HLA-DR and -DQ alleles conferring susceptibility to pollen allergy and pollen associated food allergy. Clin Exp Allergy 1998; 28:434–441.
32 Mannering SI, Morris JS, Jensen KP, Purcell AW, Honeyman MC, van Endert PM, Harrison LC: A sensitive method for detecting proliferation of rare autoantigen-specific human T cells. J Immunol Methods 2003; 283:173–183.
33 Schenk S, Breiteneder H, Susani M, Najafian N, Laffer S, Duchene M, Valenta R, Fischer G, Scheiner O, Kraft D, et al: T-cell epitopes of Phl p 1, major pollen allergen of Timothy grass *(Phleum pratense)*: evidence for cross-reacting and non-crossreacting T-cell epitopes within grass group I allergens. J Allergy Clin Immunol 1995;96:986–996.
34 Spiegelberg HL, Beck L, Stevenson DD, Ishioka GY: Recognition of T cell epitopes and lymphokine secretion by rye grass allergen *Lolium perenne* I-specific human T cell clones. J Immunol 1994;152:4706–4711.
35 Sone T, Dairiki K, Morikubo K, Shimizu K, Tsunoo H, Mori T, Kino K: Identification of human T cell epitopes in Japanese cypress pollen allergen, Cha o 1, elucidates the intrinsic mechanism of cross-allergenicity between Cha o 1 and Cry j 1, the major allergen of Japanese cedar pollen, at the T cell level. Clin Exp Allergy 2005;35:664–671.
36 Muller WD, Karamfilov T, Fahlbusch B, Vogelsang H, Jager L: Analysis of human T cell clones reactive with group V grass pollen allergens. Int Arch Allergy Immunol 1994;105: 391–396.
37 Woodfolk JA: T-cell responses to allergens. J Allergy Clin Immunol 2007;119:280–294; quiz 295–286.