

Original article

Characterization of the human T cell response to rye grass pollen allergens Lol p 1 and Lol p 5

Background: Knowledge of dominant T cell epitopes of major allergens recognized by allergic individuals is required to improve efficacy and safety of allergen immunotherapy. Rye grass pollen (RGP) is the most important source of seasonal aeroallergens in temperate climates and Lol p 1 and Lol p 5 are the two major IgE-reactive allergens. This study aimed to characterize the T cell response to these allergens using a large panel of RGP-sensitive individuals.

Methods: Short-term RGP-specific T cell lines (TCL) were generated from 38 RGP-sensitive subjects and stimulated with Lol p 1 and/or Lol p 5 allergens and synthetic 20-mer peptides. Proliferative responses were determined by ³H-thymidine uptake and IL-5 and IFN- γ in culture supernatants analysed by ELISA.

Results: Of 17 subjects tested for reactivity to both allergens 16 (94%) responded to Lol p 1 and/or Lol p 5, establishing these as major T cell-reactive allergens. Sites of T cell reactivity were spread throughout the allergen molecules but regions of high reactivity were found. For Lol p 1 these spanned residues 19–38, 109–128, 154–173, 190–209, and for Lol p 5 37–56, 100–119, 145–164, 154–173, 190–209, 217–236 and 226–245. IL-5 and IFN- γ were produced by T cells cultured with proliferation-inducing peptides.

Conclusions: T cell responses to RGP major allergens have been extensively characterized, providing fundamental information for developing T cell-targeted immunotherapy for RGP allergy.

**M. D. Burton¹, L. Papalia¹,
N. P. Eusebius^{1,2}, R. E. O'Hehir^{1,2},
J. M. Rolland¹**

¹Department of Pathology and Immunology, Monash University Medical School; ²Department of Allergy, Asthma & Clinical Immunology, Alfred Hospital and Monash University, Victoria, Australia

Key words: allergens; Lol p 1; Lol p 5; rye grass pollen; T cell epitopes.

Associate Professor Jennifer M. Rolland
Department of Pathology and Immunology
Monash University Medical School
Commercial Road
Prahran
3181 Victoria
Australia

Accepted for publication 2 July 2002

Allergen-specific CD4⁺ T cells producing Th2-type cytokines play a major role in the elicitation and progression of allergic diseases (1). Upon appropriate presentation of allergen peptide these T cells produce a cocktail of cytokines, most importantly IL-4, which drives IgE class switching by allergen-stimulated B cells, and IL-5 which plays an important role in the maturation and activation of eosinophils. Allergen immunotherapy has been successfully used to specifically treat allergic disease (2), yet the precise mechanisms involved are as yet not fully understood. Moreover, efficacy for treatment of common allergic diseases, such as those induced by grass pollens and house dust mite, is only 60–80% and there is a risk of IgE-mediated side-effects from injection of the current crude extracts (2). Several studies have shown that clinically effective immunotherapy induces an alteration in allergen-specific T cell cytokine phenotype from a predominant Th2/Th0-type to a more Th1/Th0-type (reviewed in (3)). Since the nature of the T cell response

is pivotal in determining whether clinical tolerance or allergy follows allergen challenge, T cells are obvious targets for new immunotherapy strategies.

Based on a knowledge of dominant T cell epitopes of allergens, short peptides or recombinant hypoallergenic forms of allergens are exciting effective alternatives to current immunotherapy allergen extracts. Since these new preparations cannot cross-link mast-cell-bound IgE, they should also be associated with improved safety of immunotherapy treatment. Peptide immunotherapy has already been successfully clinically trialled for the major cat allergen Fel d 1 (4–6) and the major bee venom allergen phospholipase A2 (PLA₂) (7). In addition, strategies for the generation of hypoallergenic recombinant allergens by site-directed mutagenesis have been developed for birch pollen, Timothy grass pollen and house dust mite allergens (8–10).

Rye grass pollen (RGP) is an important aeroallergen source in cool temperate climates during the grass flowering season. A soluble extract of RGP contains at least 17 allergenic proteins as identified by immunoblotting with patient serum IgE, ranging in size from 11 to 89 kDa (11, 12). Lol p 1 and Lol p 5 are the major allergens of RGP, which together are recognized by

Abbreviations: APC antigen presenting cell, EAST enzyme allergosorbent test, Lol p *Lolium perenne*, mAb monoclonal antibody, PBMC peripheral blood mononuclear cell, RGP rye grass pollen, SI stimulation index, TCL T cell line, TCC T cell clone.

almost all sera from RGP allergic donors. Limited mapping of T cell epitopes of Lol p 1 and Lol p 5 using PBMC, T cell lines (TCL) or T cell clones (TCC) has been reported previously (13–18). However, only one or a small series of donors were used in these studies and de Lalla et al. (18) used a computer-generated algorithm to identify T cell epitopes of Lol p 5.

Here we present an extensive analysis of the T cell response to the major RGP allergens Lol p 1 and Lol p 5. Using short-term RGP-specific TCL generated from PBMC of a large panel of RGP-allergic individuals, the importance of Lol p 1 and Lol p 5 as major T cell antigens of RGP was established, and several dominant T cell reactive regions were identified in both molecules. Together this information will aid in the development of more refined immunotherapy strategies for the treatment of RGP-induced allergic disease.

Materials and methods

Subjects

Thirty-eight RGP-sensitive individuals with allergic rhinitis and/or asthma (19) were recruited from the Alfred Hospital Asthma and Allergy Clinic, Melbourne, Australia. Donors were selected on the basis of a history of clinical symptoms of RGP allergy, positive RGP-specific IgE (Kallestad Allercoat Sanofi-Pasteur Diagnostics, EAST score ≥ 3) and positive skin prick test (wheal diameter ≥ 5 mm). The study was approved by the Alfred Hospital Ethics Committee, and informed consent was obtained from all donors before blood was obtained.

Antigens

Crude RGP (*Lolium perenne*) extract was prepared by gentle mixing of RGP grains (Greer, NC, USA) in PBS at 4°C overnight. The pollen extract was then centrifuged to pellet insoluble material, and the supernatant removed and sterilized by passage through a 0.2- μ m filter.

Lol p 1 was purified from crude RGP extracts by SDS-PAGE as previously described (20). Lol p 5 was purified by either SDS-PAGE as for Lol p 1 or by affinity chromatography. For this, an anti-Lol p 5 monoclonal antibody (mAb) FMC A7 (21) conjugated to Sepharose 4B (Pharmacia, Uppsala, Sweden) was used. Briefly, RGP extracts were equilibrated with 10 mM Tris pH 7.5 and passed through the affinity column. Unbound RGP extract was removed by washing with 10 mM Tris pH 7.5. Bound Lol p 5 was then eluted from the column with 0.1 M glycine pH 2.8 and the eluate immediately neutralized with 2 M Tris pH 7.0. The purified Lol p 5 (both SDS-PAGE and affinity purified) was dialyzed against PBS and filter sterilized.

Lol p 1 and 5 peptides (20-mers, 11 amino acid overlap) were purchased from Mimotopes, Clayton, Australia. Peptide sequences were based on those published for Lol p 1 A (240 amino acids) (13) and Lol p 5 A (276 amino acids) (22).

Mitogenicity of RGP, Lol p 1, Lol p 5 and peptides was excluded by culturing with oligoclonal house dust mite (HDM)- or low ammoniated latex (LAL)-reactive CD4⁺ T cells in the presence of irradiated autologous PBMC, and toxicity was excluded by co-culture of antigens with HDM- or LAL-reactive T cells in the presence of IL-2 (data not shown).

Generation of short-term RGP-specific TCL

PBMC were cultured in 24-well plates (Costar, MA, USA) at 2.5×10^6 cells/well with RGP extract at a concentration of 25 or 50 μ g/ml in RPMI 1640 medium (Gibco BRL, NY, USA) supplemented with 2 mM GlutaMAX I (Gibco BRL), 0.05 mg/ml gentamicin (David Bull Laboratories, Mulgrave, Victoria, Australia) and 5% heat-inactivated human AB⁺ serum (Sigma, MO, USA) for 7 days at 37°C in a 5% CO₂ humidified incubator. At day 7, cells were washed once and restimulated in 24-well plates at 5×10^5 cells/well with 25 or 50 μ g/ml of RGP extract in the presence of 5×10^5 cells/well irradiated (3000 rads) autologous PBMC as a source of antigen presenting cells (APC). At day 2 following restimulation, 10 U/ml of Lymphocult T-LF (LC; Biotest, Germany) was added as a source of IL-2 and at day 4, 1 ml of culture medium was removed and replaced with fresh medium and 10 U/ml of LC. For some experiments due to low cell numbers at 2 weeks, 3 week TCL were generated by restimulation with antigen and LC as above for a further week. In all experiments T cells were rested for 6–7 days after the last addition of antigen and APC before testing.

TCL proliferation assays

T cells (5×10^4 /well) were cultured in 96-well U bottom plates with either RGP extract at concentrations ranging from 25 to 200 μ g/ml, purified Lol p 1 (0.12–2 μ g/ml) or Lol p 5 (0.1–10 μ g/ml), or Lol p 1 or Lol p 5 peptides (10 μ g/ml), or medium alone (to determine background levels of proliferation), in the presence of washed autologous irradiated (3000 rads) PBMC (5×10^4 /well). For some TCL, there were insufficient cells to permit testing to both Lol p 1 and Lol p 5 peptide series. Cultures were incubated for 3 days, pulsed for the last 16 h with ³H-thymidine (1 μ Ci/well) and harvested onto glass fibre filters. ³H-thymidine incorporation was measured by liquid scintillation spectroscopy and the mean counts per minute (cpm) for triplicate cultures was determined. Responses were considered positive if the stimulation index (SI, cpm of antigen-stimulated T cells divided by cpm of unstimulated T cells) was ≥ 2.5 . Where several concentrations of allergen were tested (i.e., RGP, Lol p 1 and Lol p 5), values corresponding to the highest proliferation are shown.

Cytokine detection in culture supernatants

Supernatants of triplicate cultures were harvested and pooled from T cell proliferation assay cultures at 48 h for the detection of IL-5 and IFN- γ production by sandwich ELISA. Briefly white Maxi-sorp (NUNC, Roskilde, Denmark) ELISA plates were coated with capture mAb (anti-IL-5, Pharmingen, San Diego, CA, USA; anti-IFN- γ , Endogen, Woburn, MA, USA) overnight at 4°C. Plates were then washed and wells blocked with 1% Bovine Serum Albumin/PBS for 1 h at room temperature. Following washing, test samples (neat) and serial dilutions of recombinant human IL-5 (Pharmingen, USA) or IFN- γ (Endogen, USA) (for the construction of standard curves) were added and incubated overnight at 4°C. Following washing, plates were incubated with biotinylated detecting mAb (anti-IL-5, Pharmingen, USA; anti-IFN- γ , Endogen, USA) for 1 h at room temperature. Plates were then washed and incubated with streptavidin-peroxidase (Amersham, Piscataway, NJ, USA) for 30 min at room temperature. Following washing, a chemiluminescent substrate (Du Pont, Wilmington, DE, USA) was added to each well and plates read in a Lumicount microplate Glow Luminometer (Packard Instrument Company, Meriden, CT, USA), 0.5 s/well, automatic sensitivity setting. Standard curve construction and determination of test sample cytokine levels were performed using Packard I-smart software.

The limits of detection for IL-5 and IFN- γ were 10 pg/ml and 2 pg/ml, respectively.

Results

T cell response to RGP allergens

Stimulation of TCL with crude RGP extract induced good proliferation responses in all except two TCL (Tables 1 and 2). For these two TCL, the stimulation index was below the arbitrary cut-off of 2.5. In both these cases Lol p 5 peptide reactivity was demonstrated, suggesting that the poor response to crude RGP extract may have been due to the use of a suboptimal dose. Only one concentration of RGP was used for these subjects due to limited cell numbers. Ten of 14 (71%) TCL tested responded to purified Lol p 1 and nine of 15 (60%) TCL responded to purified Lol p 5. However, if allergen responsiveness was considered in terms of response to allergen or peptide, 79% of TCL responded to Lol p 1 and 91% to Lol p 5. These results demonstrate the importance of these two allergens at the T cell level. The fine specificity of Lol p 1 and Lol p 5 reactive T cells was revealed by comparison of responses to the nested sets of peptides.

TCL responses to Lol p 1 peptides

Twenty-four of 34 (71%) RGP-specific TCL responded to one or more Lol p 1 peptides. The proliferative responses of peptide-responsive RGP-specific TCL are shown in Table 1. With the exception of four Lol p 1 peptides (55–74, 163–182, 199–218, 208–227) all were recognized by one or more TCL. When a responder frequency analysis was performed, four peptides were recognized by 25% or more Lol p 1 peptide-responsive donors (Fig. 1). These peptides encompassed regions 19–38, 109–128, 154–173 and 190–209 of the Lol p 1 amino acid sequence. All four peptides were distinct regions of Lol p 1 indicating that each contains separate and discrete T cell epitopes. The highest frequency of donor recognition of a single peptide was 46% for Lol p 1 (19–38). Taken together the four most frequently recognized Lol p 1 peptides were recognized by 75% of Lol p 1 peptide-responsive RGP-specific TCL.

TCL responses to Lol p 5 peptides

T cell responsiveness to Lol p 5 peptides was detected in 15 of 21 (71%) TCL tested, with determinants spread throughout the central region of the molecule (Table 2). No donors responded to peptides encompassing the N- and C-terminus of Lol p 5 (residues 1–36 and 246–276, respectively), nor were T cell responses detected towards peptides 82–101, 91–110, 109–128, 127–146 or 136–155. Of the 30 overlapping Lol p 5 peptides only 17 (57%) were recognized by one or more RGP-specific TCL. As

observed for the TCL responses to Lol p 1 peptides, several TCL responded to adjacent overlapping peptides, suggesting that the responses may either be to separate T cell epitopes or to a common T cell epitope encompassed by both peptides. Of the 17 positively recognized peptides, seven were recognized by more than 25% of the peptide-responsive TCL, these being peptides 37–56, 100–119, 145–164, 154–173, 190–209, 217–236 and 226–245 (Fig. 1). The peptides to which the highest frequency of responses were directed were peptides 37–56 and 154–173, each being recognized by more than 50% of peptide-responsive donors. Together these two peptides were recognized by 80% of peptide-responsive donors. For the seven dominant Lol p 5 peptides, 93% of peptide-responsive donors recognized one or more of these peptides, however, the same recognition frequency could also be achieved with only four (37–56, 100–119, 154–173, 217–236) of these seven dominant peptides.

Production of IL-5 and IFN- γ by RGP-specific TCL

The production of IL-5 and IFN- γ was tested in 12 RGP-specific TCL (Table 3). The IL-5 and IFN- γ responses were determined for selected T cell reactive and non-reactive peptides based on proliferation assays. Following stimulation with T cell reactive Lol p 1 peptides, cytokine profiles varied, with some peptides inducing the production of both IL-5 and IFN- γ and others inducing the secretion of IFN- γ only. T cell reactive Lol p 5 peptides generally induced the production of both cytokines with higher levels of IL-5. IL-5 was rarely detected in supernatants from cultures that were incubated with peptides that failed to stimulate T cell proliferation, and similarly the levels of IFN- γ production in these cultures were minimal or undetectable.

Discussion

The importance of the RGP allergens Lol p 1 and Lol p 5 in seasonal allergy has been substantiated by several serological studies, which have shown between 84 and 100% of RGP-sensitive donors possess specific IgE for these allergens (11, 23–25). However, there is currently limited information on the T cell reactivity of these allergens (13–18, 26). Thus, a more comprehensive investigation into the specificity and quality of the T cell response to Lol p 1 and Lol p 5 is important if the development of refined immunotherapy for RGP allergy is to proceed. In this study, Lol p 1 and 5 were both shown to be major T cell antigens of RGP with recognition frequencies of 79% and 91%, respectively, and both molecules were shown to have several highly T cell reactive regions.

RGP-specific TCL generated from the PBMC of a large panel of RGP allergic individuals recognized multiple T cell epitopes in both the Lol p 1 A and 5 A sequences, consistent with the findings for other allergens (reviewed in (27)).

Table 1. Lol p 1 epitope specificities of RGP oligoclonal TCLs

Peptide	Subject																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1–20																								2.6
10–29				3.1									3.0											
19–38		5.2	3.3	12.3		2.7	3.2	3.8				2.5			10.5		5.3		10.3		3.8			
28–47				4.1							3.5				2.6				3.0					
37–56				2.5																2.6				
46–65					8.4							4.0												
55–74																								
64–83	7.2														3.5									
73–92	3.9			2.9							2.6													
82–101											3.6													
91–110				3.6							4.9													
100–119		9.4		6.8							7.9													
109–128		12.9	2.8									2.5			9.2		2.7		3.7					
118–137		6.2													7.1	3.0			12.0					
127–146		5.8											3.7				3.0							
136–155				2.5										4.4										
145–164												4.5												
154–173				3.0					3.0		4.2	3.0			3.1								4.2	
163–182																								
172–191									2.5															
181–200				4.5							7.3													
190–209				6.2						3.7	3.7				5.8		2.6		3.0				6.2	
199–218															5.1		5.4				5.7		5.2	
208–227																								
217–236											4.4													
221–240											3.3													
RGP	9.7	9.3	14.4	22.1	9.9	15.3	4.0	6.8	7.0	5.6	10.0	3.5	3.0	5.6	20.2	12.9	19.3	3.9	19.7	19.1	30.5	20.0	15.2	14.2
Lol p 1	5.9	3.0	NT	2.9	32.5	4.0		NT		NT	NT	NT	NT	NT	3.3	NT			10.5	NT	NT	NT	NT	NT

Proliferative responses for Lol p 1 peptide-responsive TCL are shown as stimulation indices. Only responses considered positive, i.e., where stimulation indices were 2.5 or greater, are shown. Background responses of T cells cultured with irradiated APC in the absence of antigen were 2121 ± 2628 cpm (mean $\pm$ SD). The maximal RGP and Lol p 1 response is shown for each donor. NT, not tested.

Table 2. Lol p 5 peptide specificities of RGP oligoclonal TCLs

Peptide	Subject														
	2	4	6	7	8	11	12	13	20	27	29	35	36	37	38
1–20															
10–29															
19–38															
28–47															
37–56	4.3		2.7			4.6		3.9	4.9		2.8	4.1			4.0
46–65													2.7	6.6	
55–74													3.3		3.3
64–83		2.8		2.8											
73–92				2.6											
82–101															
91–110															
100–119		3.8				10.0	7.5					2.5			
109–128															
118–137									4.5	2.9					
127–146															
136–155															
145–164		3.1						6.4	5.3		4.5	5.3			
154–173	3.8	4.6			5.2	4.5				2.5		4.1		6.4	4.5
163–182									2.5						
172–191		2.7					2.5		3.0						
181–200									3.4						
190–209	3.0	5.1				4.0					3.1				
199–218		2.5				5.6								4.8	
208–227						4.3			4.6				2.6		
217–236						5.4			4.5	9.5		3.5	3.5	11.8	
226–245	3.3					3.5		2.9		7.1					
235–254															
244–263															
253–272															
257–276															
Lol p 5		4.9*			4.3†	9.4†		NT	NT	3.8†	NT	NT	NT	NT	3.2*
RGP	5.7	5.2	19.3	3.0	12.3	12.8	7.0		4.8	3.2	7.8		7.6	3.6	5.3

Proliferative responses for Lol p 5 peptide-responsive TCL are shown as stimulation indices. Only responses considered positive, i.e., where stimulation indices were 2.5 or greater, are shown. Background responses of T cells cultured with irradiated APC in the absence of antigen were 2720 ± 2971 cpm (mean $\pm$ SD). The maximal RGP and Lol p 5 response is shown for each donor.

NT, not tested.

*SDS-PAGE purified Lol p 5.

†Affinity purified Lol p 5.

Four Lol p 1 peptides (19–38, 109–128, 154–173, 190–209) and seven Lol p 5 peptides (37–56, 100–119, 145–164, 154–173, 190–209, 217–236, 226–245) were recognized by 25% or more peptide-responsive donor TCL. To clearly define core T cell epitopes within these peptides, a series of shorter peptides or N- and C-terminal truncated peptides would be required. Cytokine responses to peptides generally paralleled proliferative responses, and as we have previously reported for Bermuda grass pollen allergen Cyn d 1 peptides, immunodominant Lol p 1 and Lol p 5 peptides induced production of IL-5 and IFN- γ by short-term TCL from patients who had not received immunotherapy (28). IL-4 production was not investigated since we find only very low or undetectable IL-4 levels by ELISA in such short-term TCL supernatants.

Previous studies of the T cell epitopes of Lol p 1 (13–16) revealed a region encompassed by residues 191–210 to be the most frequently recognized, and they thus

harbour a major T cell epitope of Lol p 1. Similarly we found high T cell reactivity towards this region (peptides 190–209), although it was only the second most frequently recognized peptide of the Lol p 1 series. Interestingly we found the most dominant region to be encompassed by residues 19–38, a region of low T cell reactivity in previous studies. This conflicting finding is most likely due to the limited number of donors used in the other studies, and also to the use of TCC (13, 14). The two other dominant regions of Lol p 1 identified here (109–128 and 154–173) were previously found to induce low to moderate T cell reactivity (14–16).

The regions encompassing the most frequently recognized Lol p 5 epitopes include those identified in two previous studies (17, 18), although the study by de Lalla and colleagues (18) was not a conventional T cell epitope mapping study. Using an algorithm (TEPITOPE) they identified four promiscuous HLA-DR binding peptides of Lol p

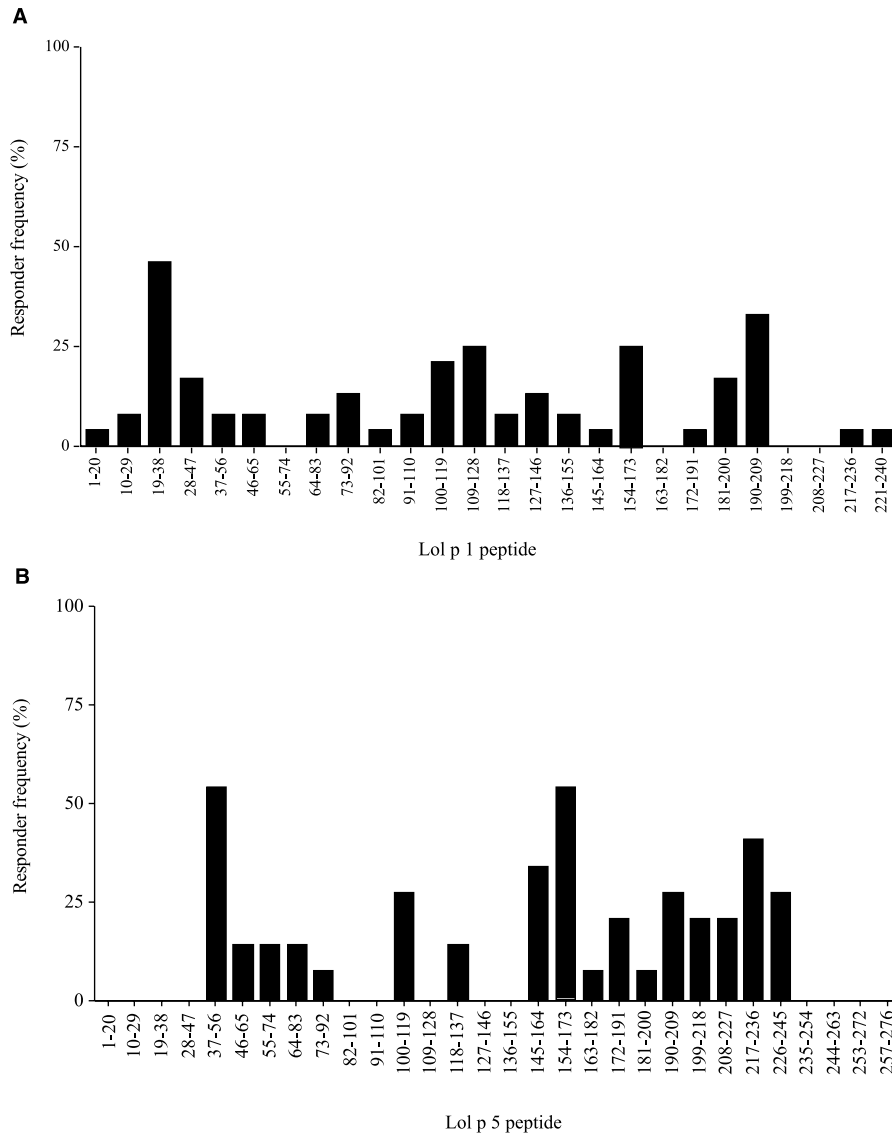


Figure 1. Responder frequency of TCL from a panel of 34 atopic donors to individual Lol p 1 peptides (A) and from a panel of 21 atopic donors to individual Lol p 5 peptides (B). A stimulation index of ≥ 2.5 was considered positive and the number of donor TCL responding to each peptide is shown as a percentage.

5 A (56–68, 109–121, 219–231, 224–236) and demonstrated that these were T cell reactive, using TCC generated against a pool of the four peptides (18). The peptide encompassing residues 56–68 was recognized at moderate frequency by our subjects, and residues 219–231 and 224–236 are encompassed by the dominant peptides 217–236 and 226–245 identified here. However our study did not support peptide 109–121 as containing an important T cell epitope of Lol p 5, despite an overlapping peptide 100–119 being recognized at high frequency. In fact peptide 109–128 was not recognized by any donor. This result is consistent with our previous identification of residue 107 (Y) as a critical MHC binding residue of the core Lol p 5 T cell epitope 107–114 (29). Although de Lalla et al. demonstrated that two of the peptide-generated TCC responded to peptide 109–121,

they did not respond to RGP extract, suggesting that this peptide is not generated by natural Lol p 5 processing. The two epitopes of Lol p 5 previously identified by Blaher et al. (17) (105–116 and 193–204), are encompassed by the peptides 100–119 and 190–209 identified as dominant in this study. That most of the Lol p 5 epitopes identified in the two previous studies were also identified here in this study as regions with high recognition frequencies supports the conclusion that these regions encompass major T cell epitopes of Lol p 5 and are thus suitable for refined immunotherapy strategies. However our larger study has also identified distinct T cell epitopes of Lol p 5 not described previously (37–56, 145–164, 154–173). These include the two most frequently recognized peptides, 37–56 and 154–173. Why such frequently recognized epitopes

Table 3. IL-5 and IFN- γ production by RGP-specific TCL stimulated with Lol p 1 or Lol p 5 peptides

Subject	Proliferation inducing peptides			Non-proliferation inducing peptides	
	Peptide	IL-5 (pg/ml)	IFN- γ (pg/ml)	IL-5 (pg/ml)	IFN- γ (pg/ml)
Lol p 1 peptides					
3	19–38	UD	20.9	UD	5.7
	109–128	UD	32.1		
10	190–209	UD	9.0	UD	5.4
14	136–155	UD	106.4	UD	24.9
15	19–38	182.7	204.1	UD	24.5
	28–47	UD	23.2		
	64–83	26.5	38.8		
	100–119	275.6	167.1		
	109–128	160.7	143.3		
	154–173	26.6	83.9		
	181–200	13.3	56.6		
	190–209	19.3	63.5		
16	109–128	UD	13.8	UD	5.1
19	19–38	UD	42.8	UD	0.6
	28–47	UD	5.2		
	100–119	UD	UD		
	109–128	UD	8.3		
	190–209	14.7	UD		
20	37–56	46.9	11.1	UD	5.9
23	154–173	12.4	11.2	3.9	4.6
	181–200	25.1	11.7		
	190–209	UD	8.3		
24	1–20	46.1	UD	UD	UD
Lol p 5 peptides					
2	37–56	692.8	317.0	UD	38.3
	154–173	546.3	400.3		
	190–209	518.1	87.8		
	226–245	UD	292.0		
38	37–56	275.1	85.0	12.2	27.6
	55–74	220.7	54.2		
	154–173	256.3	198.1		

Supernatants were harvested at 48 h from T cell cultures and tested by ELISA for the presence of IL-5 and IFN- γ . Cytokine levels induced by immunoreactive peptides are shown on the left of the table and as mean values for unreactive peptides on the right.

UD, undetectable.

were not identified in the two previous studies is probably due to the use of a single donor in the study by Blaher et al. and of only promiscuous HLA-DR binding ligands which lay within a specific group 5 allergen motif being tested by de Lalla and colleagues.

When considering T cell epitope based immunotherapy strategies using peptides, questions on the ability of an individual peptide to bind a range of different MHC haplotypes as seen in outbred populations are raised. However evidence exists for peptides binding to a wide variety of MHC alleles (30–32) due to similar MHC peptide binding motifs or 'supermotifs' (33–35). Indeed the region encompassing a dominant T cell epitope of PLA₂, used in peptide-immunotherapy for the treatment of bee venom allergy, has been shown to bind efficiently all

seven of the predominant HLA-DR alleles expressed in the Caucasian population (36). Although the HLA haplotypes of the patients used in this study were not determined, the high frequency of recognition for the dominant Lol p 1 and 5 peptides, especially peptides 19–38 of Lol p 1 and 37–56 and 154–173 of Lol p 5, indicates that the encompassed epitopes are likely to be promiscuous, binding to a wide variety of HLA alleles. That some of the dominant Lol p 5 peptides correspond to regions where highly promiscuous epitopes of Lol p 5 for HLA-DR have been identified (18) further supports this conclusion.

The finding that some donor TCL did not respond to any peptide but did to the respective purified allergen suggests either that inappropriate doses were tested due to limited cell numbers or that the T cell specificities of some individuals may be directed towards specific allergen isoforms. Differential T cell recognition of allergen isoforms has been demonstrated for other allergens (37–42) and is a significant hurdle that must be addressed when considering T cell targeted immunotherapies. Both Lol p 1 and Lol p 5 exist in several isoforms, four of which are known to exist for Lol p 1 and eight for Lol p 5 (43). Three isoforms of each allergen have been cloned to date. The Lol p 1 isoforms are highly conserved at the amino acid sequence level (13, 44), whereas the Lol p 5 isoforms are more heterogeneous (22, 24, 25). Of importance is whether the sequence variations occur at critical residues for MHC binding or T cell receptor engagement. However, since such a high frequency of subjects recognize several peptides for each of Lol p 1 and Lol p 5, it is likely that sequence variations in general are not important and/or that the different isoforms are presented to and recognized by T cells of the majority of donors. Our previous studies on the dominant Lol p 5 epitope encompassed by residues 100–119 indicate that isoform sequence variations would not be expected to affect critical residue function (29).

In conclusion, we have shown that Lol p 1 and Lol p 5 are major T cell antigens of RGP and are thus suitable allergens against which new RGP immunotherapy strategies could be targeted. T cell reactive regions of Lol p 1 and Lol p 5 were identified, with several regions containing dominant epitopes, being recognized by $\geq 25\%$ of peptide-responsive donors. Peptides or recombinant modified allergens based on these dominant T cell epitopes of Lol p 1 and Lol p 5 would be safer due to no or little potential for cross-linking of cell-bound IgE. By specifically targeting T cells they would also be expected to be of greater efficacy for the treatment of RGP-mediated allergic disease symptoms.

Acknowledgments

This work was supported by the National Health and Medical Research Council of Australia, The Alfred Research Trusts and the Australian Allergy Foundation.

References

- ROMAGNANI S. The Th1/Th2 paradigm. *Immunol Today* 1997;**18**:263–266.
- BOUSQUET J, LOCKEY RF, MALLING HJ. WHO Position Paper; Allergen immunotherapy: therapeutic vaccines for allergic diseases. *Allergy* 1998;**53**:1–42.
- ROLLAND J, O'HEHIR R. Immunotherapy of allergy: anergy, deletion, and immune deviation. *Curr Opin Immunol* 1998;**10**:640–645.
- PENE J, DESROCHES A, PARADIS L et al. Immunotherapy with Fel d 1 peptides decreases IL-4 release by peripheral blood T cells of patients allergic to cats. *J Allergy Clin Immunol* 1998;**102**:571–578.
- ALEXANDER C, OLDFIELD WLG, SHIRLEY K, LARCHE M, KAY AB. Dosing protocol of allergen-derived T cell peptide epitopes for the treatment of allergic disease. *J Allergy Clin Immunol* 2001;**102**:S217.
- OLDFIELD WLG, SHIRLEY K, LARCHE M, KAY AB. A double-blind, placebo-controlled study of short peptides derived from Fel d 1 in cat allergic subjects. *J Allergy Clin Immunol* 2001;**107**:S216.
- MULLER U, AKDIS CA, FRICKER M et al. Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom. *J Allergy Clin Immunol* 1998;**101**:747–754.
- VRTALA S, HIRTENLEHNER K, SUSANI M et al. Genetic engineering of recombinant hypoallergenic oligomers of the major birch pollen allergen, Bet v 1: candidates for specific immunotherapy. *International Arch Allergy Immunol* 1999;**118**:218–219.
- SCHRAMM G, KAHLERT H, SUCK R et al. "Allergen engineering": variants of the timothy grass pollen allergen Phl p 5b with reduced IgE-binding capacity but conserved T cell reactivity. *J Immunol* 1999;**162**:2406–2414.
- SMITH AM, CHAPMAN MD, TAKETOMI EA, PLATTS-MILLS TA, SUNG SS. Recombinant allergens for immunotherapy, a Der p 2 variant with reduced IgE reactivity retains T-cell epitopes. *J Allergy Clin Immunol* 1998;**101**:423–425.
- FORD SA, BALDO BA. A re-examination of ryegrass (*Lolium perenne*) pollen allergens. *Int Arch Allergy Appl Immunol* 1986;**81**:193–203.
- STEWART GA, TURNER KJ, BALDO BA et al. Standardization of rye-grass pollen (*Lolium perenne*) extract. An immunochemical and physicochemical assessment of six candidate international reference preparations. *Int Arch Allergy Appl Immunol* 1988;**86**:9–18.
- PEREZ M, ISHIOKA GY, WALKER LE, CHESNUT RW. cDNA cloning and immunological characterization of the rye grass allergen Lol p I. *J Biol Chem* 1990;**265**:16210–16215.
- 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.
- BUNGY POOR FARD GA, LATCHMAN Y, RODDA S, GEYSEN M, ROITT I, BROSTOFF J. T cell epitopes of the major fraction of rye grass *Lolium perenne* (Lol p I) defined using overlapping peptides in vitro and in vivo. I. Isoallergen clone 1A. *Clin Exp Immunol* 1993;**94**:111–116.
- BUNGY GA, RODDA S, ROITT I, BROSTOFF J. Mapping of T cell epitopes of the major fraction of rye grass using peripheral blood mononuclear cells from atopics and non-atopics. II. Isoallergen clone 5A of *Lolium perenne* group I (Lol p I). *Eur J Immunol* 1994;**24**:2098–2103.
- BLAHER B, SUPHIOGLU C, KNOX RB, SINGH MB, MCCLUSKEY J, ROLLAND JM. Identification of T-cell epitopes of Lol p 9, a major allergen of ryegrass (*Lolium perenne*) pollen. *J Allergy Clin Immunol* 1996;**98**:124–132.
- DE LALLA C, STURNIOLO T, ABBRUZZESE L et al. Cutting edge: identification of novel T cell epitopes in Lol p 5a by computational prediction. *J Immunol* 1999;**163**:1725–1729.
- JOHANSSON SGO J'OB, BOUSQUET J, BRUIJNZEEL-KOOMEN C, et al. A revised nomenclature for allergy. *Allergy* 2001;**56**:813–824.
- LEVY D, DAVIES J, O'HEHIR R, SUPHIOGLU C. A rapid and efficient two-step gel electrophoresis method for the purification of major rye grass pollen allergens. *Electrophoresis* 2001;**22**:1900–1905.
- SMART IJ, HEDDLE RJ, ZOLA H, BRADLEY J. Development of monoclonal mouse antibodies specific for allergenic components in ryegrass (*Lolium perenne*) pollen. *Int Arch Allergy Appl Immunol* 1983;**72**:243–248.
- ONG EK, GRIFFITH IJ, KNOX RB, SINGH MB. Cloning of a cDNA encoding a group-V (group-IX) allergen isoform from rye-grass pollen that demonstrates specific antigenic immunoreactivity. *Gene* 1993;**134**:235–240.
- FREIDHOFF LR, EHRLICH-KAUTZKY E, GRANT JH, MEYERS DA, MARSH DG. A study of the human immune response to *Lolium perenne* (rye) pollen and its components, Lol p I and Lol p II (rye I and rye II). I. Prevalence of reactivity to the allergens and correlations among skin test, IgE antibody, and IgG antibody data. *J Allergy Clin Immunol* 1986;**78**:1190–1201.
- SINGH MB, HOUGH T, THEERAKULPISUT P et al. Isolation of cDNA encoding a newly identified major allergenic protein of rye-grass pollen: intracellular targeting to the amyloplast. *Proc Nat Acad Sci USA* 1991;**88**:1384–1388.
- SUPHIOGLU C, MAWDSLEY D, SCHAPPI G et al. Molecular cloning, expression and immunological characterisation of Lol p 5C, a novel allergen isoform of rye grass pollen demonstrating high IgE reactivity [In Process Citation]. *FEBS Lett* 1999;**462**:435–441.
- BLAHER B, MCCLUSKEY J, PUY R, CZARNY D, ROLLAND JM. T cell response to grass pollen allergens: correlation with skin test reactivity and serum IgE levels. *Immunol Cell Biol* 1995;**73**:17–22.
- VAN NEERVEN RJ, EBNER C, YSSEL H, KAPSENBERG ML, LAMB JR. T-cell responses to allergens: epitope-specificity and clinical relevance. *Immunol Today* 1996;**17**:526–532.
- EUSEBIUS NP, PAPALIA L, SUPHIOGLU C et al. Oligoclonal analysis of the atopic T cell response to the group I allergen of *Cynodon dactylon* (Bermuda grass) pollen: pre- and post-allergen specific immunotherapy. *Int Arch Allergy Immunol* 2002;**127**:234–244.
- BURTON MD, BLAHER B, SUPHIOGLU C, O'HEHIR RE, CARBONE FR, ROLLAND JM. T-cell receptor contact and MHC binding residues of a major rye grass pollen allergen T-cell epitope. *J Allergy Clin Immunol* 1999;**103**:255–261.
- ALEXANDER J, SIDNEY J, SOUTHWOOD S et al. Development of high potency universal DR-restricted helper epitopes by modification of high affinity DR-blocking peptides. *Immunity* 1994;**1**:751–761.

31. PANINA-BORDIGNON P, TAN A, TERMIJTELEN A, DEMOTZ S, CORRADIN G, LANZAVECCHIA A. Universally immunogenic T cell epitopes: promiscuous binding to human MHC class II and promiscuous recognition by T cells. *Eur J Immunol* 1989;**19**:2237–2242.
32. SINIGAGLIA F, GUTTINGER M, KILGUS J et al. A malaria T-cell epitope recognized in association with most mouse and human MHC class II molecules. *Nature* 1988;**336**:778–780.
33. O'SULLIVAN D, ARRHENIUS T, SIDNEY J et al. On the interaction of promiscuous antigenic peptides with different DR alleles. Identification of common structural motifs. *J Immunol* 1991;**147**:2663–2669.
34. SINIGAGLIA F, HAMMER J. Motifs and supermotifs for MHC class II binding peptides. *J Exp Med* 1995;**181**:449–451.
35. SOUTHWOOD S, SIDNEY J, KONDO A et al. Several common HLA-DR types share largely overlapping peptide binding repertoires. *J Immunol* 1998;**160**:3363–3373.
36. TEXIER C, POUVELLE S, BUSSON M et al. HLA-DR restricted peptide candidates for bee venom immunotherapy. *J Immunol* 2000;**164**:3177–3184.
37. WURTZEN P, WISSENBACH M, IPSEN H, BUFE A, ARNVED J, VAN NEERVEN RJ. Highly heterogeneous Phl p 5-specific T cells from patients with allergic rhinitis differentially recognize recombinant Phl p 5 isoallergens. *J Allergy Clin Immunol* 1999;**104**:115–122.
38. VAN NEERVEN R, WISSENBACH M, IPSEN H, BUFE A, ARNVED J, WURTZEN PA. Differential recognition of recombinant Phl p 5 isoallergens by Phl p 5-specific T cells. *Int Arch Allergy Immunol* 1999;**118**:125–128.
39. MULLER WD, KARAMFILOV T, KAHLERT H et al. Mapping of T-cell epitopes of Phl p 5: evidence for crossreacting and non-crossreacting T-cell epitopes within Phl p 5 isoallergens. *Clin Exp Allergy* 1998;**28**:1538–1548.
40. FERREIRA F, HIRTENLEHNER K, JILEK A et al. Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. *J Exp Med* 1996;**183**:599–609.
41. SPARHOLT SH, LARSEN JN, IPSEN H, SCHOU C, VAN NEERVEN RJ. Crossreactivity and T-cell epitope specificity of Bet v 1-specific T cells suggest the involvement of multiple isoallergens in sensitization to birch pollen. *Clin Exp Allergy* 1997;**27**:932–941.
42. THOMAS WR, SMITH W, HALES BJ, CARTER MD. Functional effects of polymorphisms of house dust mite allergens. *Int Arch Allergy Immunol* 1997;**113**:96–98.
43. SMITH PM, ONG EK, KNOX RB, SINGH MB. Immunological relationships among group I and group V allergens from grass pollen. *Mol Immunol* 1994;**31**:491–498.
44. GRIFFITH IJ, SMITH PM, POLLOCK J et al. Cloning and sequencing of Lol p I, the major allergenic protein of rye-grass pollen. *FEBS Lett* 1991;**279**:210–215.