

Identification of isoform-specific T-cell epitopes in the major timothy grass pollen allergen, Phl p 5

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Summary

Background The involvement of CD4⁺ T cells in the pathophysiology of atopic disease is well established.

Objective To gain further insight into the activation requirements for allergen-specific T cells, we characterized epitope specificity, HLA restriction and T-cell receptor (TCR) usage for T cells specific to Phl p 5, the group 5 major allergen of the grass *Phleum pratense*.

Methods To identify the T-cell epitopes of Phl p 5, three Phl p 5-specific T-cell lines (TCLs) and 15 T-cell clones (TCCs) generated from the peripheral blood of three grass-allergic patients were tested with recombinant truncated Phl p 5a fragments and synthetic Phl p 5b peptides representing these two different recombinant Phl p 5 isoallergens. Additional activation experiments with HLA-subtyped antigen-presenting cells and flow cytometry analysis with TCR V-specific mAb were performed to further characterize the activation requirements for Phl p 5-specific TCCs.

Results At least nine distinct T-cell specificities were identified and the T-cell epitopes recognized differed considerably among the three patients. Most of the epitopes found were isoform-specific, whereas three epitopes were shared between Phl p 5a and 5b. Several human leucocyte antigen (HLA) class II molecules were involved in the recognition of Phl p 5. Different HLA restriction specificities were even found among TCCs specific to the same epitope region. All TCCs were TCR- α/β positive, and an overrepresentation of TCR V β 3.1⁺ clones among TCCs specific to Phl p 5 appear to exist as 31% (4/13) of the TCCs expressed TCR V β 3.1 (compared with 5% TCR V β 3.1⁺ T cells in human peripheral blood) with no correlation with epitope specificity or HLA restriction.

Conclusion The T-cell reactivity of the three grass-allergic patients investigated shows that isoallergen-specific T-cell epitopes are found throughout the peptide backbone of Phl p 5a and Phl p 5b, and dominant T-cell epitopes of Phl p 5 were not identified. This indicates that a mixture of at least full-length rPhl p 5a and rPhl p 5b may be required to target the total Phl p 5-specific T-cell response of atopic patients.

Keywords: grass group 5 allergens, major histocompatibility complex, T lymphocytes, T-cell epitopes, T-cell receptors

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Introduction

Type 1 allergy is a rapidly increasing health problem in the Western world, and allergic responses to house dust mite, grass and tree pollen, and animal dander are frequently observed [1]. The presence of highly polarized

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allergen-specific TH2-cell populations in the peripheral blood of atopic patients [2,3], and the preferential isolation of TH2-like T cells from the sites of allergic inflammation [4–7] have clearly demonstrated the involvement of CD4⁺ T cells in the pathophysiology of atopic diseases. The involvement of allergen-specific T cells in orchestrating the allergic response has led to the characterization of T-cell clones (TCCs) from atopic patients specific to several major allergens [8], including the major allergens from house dust mites [9,10], cat [11,12] as well as tree [13] and grass pollens [14–18]. In general, these studies have shown that allergens contain multiple T-cell epitopes, recognized by highly heterogeneous T cells with respect to specificity, human leucocyte antigen (HLA) restriction, and T-cell receptor (TCR) usage [8]. However, only a few studies [16–18] have investigated grass group 5-specific TCCs from atopic patients and the T-cell epitopes of this major grass allergen have not yet been identified. Müller *et al.* [17] suggested that the C-terminal part of Phl p 5 may harbour the majority of the T-cell epitopes on basis of the activation of Phl p 5-specific TCCs by the C-terminal peptide, but not the N-terminal half of the molecule. In addition, limited TCR V α usage [18] and HLA-DR restriction [16] have been suggested for the T-cell response to Poa p 5 and Phl p 5, respectively. As these observations indicate that a limited T-cell population is involved in the T-cell recognition of grass group 5 allergens in atopic patients, further investigation of grass group 5-specific TCCs may reveal possible targets for specific treatment of grass allergy. The immunoglobulin (Ig)E reactivity of grass-allergic patients to grass group 5 major pollen allergens is well described and this allergen group has been shown to be highly clinically relevant [19] for the frequently observed seasonal allergies to Poaceae grasses [20]. Phl p 5 is composed of two separate isoallergen groups, namely Phl p 5a and Phl p 5b visualized as two protein bands in SDS-PAGE with apparent molecular masses of 31 kDa (Phl p 5a) and 28 kDa (Phl p 5b). cDNA encoding isoallergens of both these groups have been cloned and sequenced [21–23], and sequence alignment of Phl p 5a and Phl p 5b isoforms reveals several inserts/deletions and a relatively high number of amino acid substitutions between the groups, whereas almost complete sequence identity is found among isoforms within each isoallergen group [23]. Polyclonal Phl p 5-specific serum IgE has been described to bind Phl p 5a, Phl p 5b isoallergens, and natural Phl p 5 equally well [23,24]. In contrast, we have recently shown that these isoallergens are differentially recognized by individual Phl p 5-specific T cells, and that additional as yet unidentified isoallergens seem to be involved in the T-cell recognition of this major allergen [25].

In the present investigation recombinant truncated Phl p 5a fragments and synthetic Phl p 5b peptides representing

the known Phl p 5 isoallergen sequences were used to investigate T-cell epitopes of Phl p 5 recognized by grass-allergic patients. The long recombinant Phl p 5a fragments as opposed to small synthetic peptides were chosen on the basis of the large size of Phl p 5. Furthermore, the usage of TCR V β gene products and HLA class II restriction specificity of these TCCs was investigated to describe the cognate interactions involved in the activation of Phl p 5-specific TCCs.

Materials and methods

Characterization of blood donors

Blood samples from three allergic patients were collected during the Danish grass pollen season (patient AGC: HLA class II DRB1*1302, 1501; DRB3*0301; DRB5*0101; DQA1*0102; DQB1*0602, 0604, patient TL: HLA class II DRB1*0101, 0801; DQA1*0101, 0401; DQB1*0501, 0402, and patient MSC: HLA class II DRB1*0101, 1501; DRB5*0101, DQA1*0101, 0102; DQB1*0501, 0602). The patients had a clinical history of allergy to grass pollen suffering from itching, sneezing, rhinorrhea, and mild asthma during the grass pollination season, a positive skin prick test to *Phleum pratense* pollen extract (SoluPrick SQ, ALK-Abelló, Hørsholm, Denmark), and Phl p 5-specific IgE in serum detected by immunoblotting. None of the patients had received specific immunotherapy prior to the collection of blood samples.

All patients provided written informed consent and the protocol was approved by the Danish ethic committee.

Allergens

Phleum pratense pollen extract (Phl p) Pollens from *Phleum pratense* were obtained from Greer Laboratories (Lenoir, NC, USA) and extracted as described elsewhere [2]. Working solutions (1 mg lyophilized material per mL of RPMI 1640 medium, Gibco, Paisley, UK) were sterile filtered (0.22 μ m filter, Microgen, USA) and stored at -20°C until used. The extracts contained all important allergens as evaluated by SDS-PAGE immunoblotting.

Purified Phl p 5 Phl p 5 was purified from *Phleum pratense* allergen extracts as previously described [25]. Briefly, crude extract was fractionated on a Phenyl sepharose CL-4B column (Pharmacia, Uppsala, Sweden) followed by purification by gel filtration on a S-75 superdex column (Pharmacia) according to the manufacturer's description. Working solutions (1 mg/mL), were sterile-filtered and stored at -20°C until use for T-cell stimulation.

Recombinant Phl p 5a and Phl p 5b Recombinant isoforms Phl p 5a and 5b were produced as described

elsewhere [21,22]. Briefly, genes encoding the full-length allergens were constructed by polymerase chain reaction (PCR) amplification and ligated into pQE9 expression vectors (Qiagen, Chatsworth, CA, USA). The recombinant protein material fused to a 10 amino acid N-terminal tail including a six amino acid his-tag was purified on a Ni_2^+ -NTA-resin column according to the manufacturer's instructions followed by size exclusion chromatography. Working solutions (1 mg/mL), were sterile-filtered and stored at -20°C until use for T-cell stimulation.

Truncated recombinant Phl p 5a fragments and synthetic peptides

cDNA of truncated Phl p 5a fragments was established by

rtPCR and ligated into pQE-expression vectors as described elsewhere [26]. The nucleic acid sequences of the inserts were determined by sequence analysis (Phl p 5a pep 1–5, Fig. 1). Pep 1, 2, and 5 contained sequence dissimilarities compared with Phl p 5a. Pep 1 contained amino acid substitutions in positions 29, 30, 54, 56, 60, and 74, pep 2 contained a substitution in position 74, while pep 5 contained substitutions in position 198 and a deletion of amino acids 260–264. The recombinant protein material fused to a 10 amino acid N-terminal tail including a six amino acid his-tag was expressed in *Escherichia coli* and purified on a Ni_2^+ -NTA-resin column according to the manufacturer's instructions. The resulting protein preparations were enriched in the recombinant product as evaluated by SDS-PAGE, silver-staining and immunoblotting with an

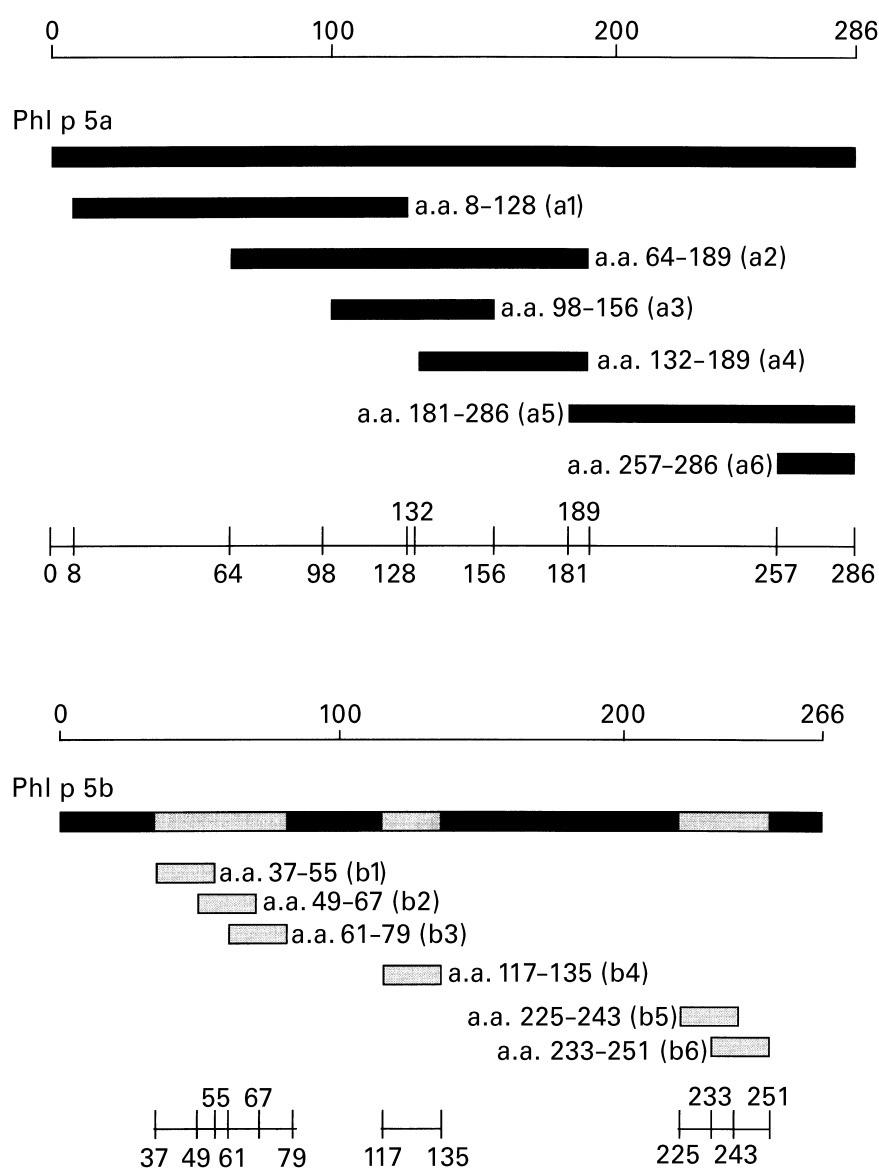


Fig. 1. Phl p 5a (upper panel) and Phl p 5b (lower panel) peptides used for stimulation of Phl p 5-specific TCLs and TCCs to identify T-cell epitopes of Phl p 5a and 5b. The peptides represent the entire Phl p 5a sequence (black), while the three unique sequence stretches of Phl p 5b were represented by overlapping synthetic peptides (grey). The subdivision of the Phl p 5a and Phl p 5b unique sequences for T-cell recognition through the use of the overlapping peptides and recombinant fragments is indicated below each panel.

anti-his-tag antibody (Qiagen). The recombinant materials were transferred to RPMI 1640 medium, sterile filtered and stored at -20°C until use for T-cell stimulation. Optimal concentrations (diluted 1:40 in all cases) for T-cell stimulation assays were determined in pilot studies using several Phl p 5-specific TCLs and TCCs. One synthetic peptide representing the C-terminal Phl p 5a sequence (Phl p 5a pep 6, Fig. 1) and six synthetic peptides representing three stretches of Phl p 5b unique sequences (Phl p 5b pep 1–6, Fig. 1) were purchased from Schafer-N (Copenhagen, Denmark).

T-cell cultures

Establishment of Phl p 5-specific T-cell lines T-cell lines specific to Phl p 5 were previously established from peripheral blood mononuclear cells (PBMCs) of three grass-allergic patients (AGC, MSC and TL) as described elsewhere [25]. In brief, isolated PBMCs ($2 \times 10^6/\text{mL}$) were stimulated in 1 mL bulk cultures in RPMI 1640 (Gibco) supplemented with 5% human AB negative serum (Harlan, Blackthorn, UK) with Phl p 5 ($20 \mu\text{g}/\text{mL}$) for 14 days, with the addition of 5–10 U r-IL2 (Chiron, Emersville, CA, USA) at days 5–7. At day 14, T cells were restimulated with autologous irradiated EBV B cells, allogeneic PBMCs, and $10 \mu\text{g}/\text{mL}$ PHA-C (Boehringer Mannheim, Mannheim, Germany). r-IL2 was added at days 5–7. The T-cell lines were kept in culture with restimulation every 10th day. The cultures were terminated after a culture period of 8 weeks, and all data presented were obtained within the first 4 weeks of culture.

Establishment of Phl p 5-specific T-cell clones On day 14 or 28 after the initial stimulation Phl p 5-specific T cells were isolated from the T-cell lines and cloned by limiting dilution as previously described [25]. The Phl p 5-specific TCCs were kept in culture with restimulation as described for TCLs every 10th day and addition of r-IL2 at days 3–5.

T-cell stimulation assay On day 10 after restimulation T cells ($2 \times 10^4/\text{well}$) were cultured with autologous or allogeneic, HLA class II typed PBMCs ($10^5/\text{well}$, irradiated 2500 Rad) in $200 \mu\text{L}$ culture medium with or without antigen in 96-well round-bottomed microtitre plates (Nunc, Roskilde, Denmark). Cells were cultured for 72 h in a humidified atmosphere at 37°C and 5% CO_2 , followed by an 18-h pulse with $0.5 \mu\text{Ci}$ ^3H -thymidine/well, and harvest for scintillation counting to determine cell proliferation. Results are expressed as mean c.p.m. values of triplicate cultures.

Flow cytometry The V β usage of the TCCs was determined

by flow cytometry analysis (Facsalibur, Becton Dickinson, Franklin Lakes, NJ, USA). T cells were labelled with a panel of FITC-conjugated mAbs specific to V β -3.1, -5.2/-5.3, -0.3, -5.1, -6.7, -8, -12, -13.1/-13.3, V α -2, or 12.3, respectively (Endogen, Woburn, MA, USA). The expression of TCR $\alpha\beta$, TCR $\gamma\delta$ and CD4/CD8 was determined through indirect staining with FITC-conjugated F(ab) $_2$ rabbit antimouse (Dako, Glostrup, Denmark) as secondary antibodies using anti TCR $\alpha\beta$ (Becton Dickinson), TCR $\gamma\delta$ (Becton Dickinson) or anti-CD4 or anti-CD8 (Ortho Diagnostic System, Raritan, NJ, USA) mAbs in the first layer.

Results

Characterization of Phl p 5-specific T-cell cultures

Three TCLs (40–60% CD3 $^{+}$ CD4 $^{+}$ CD8 $^{-}$) and 38 CD3 $^{+}$ -CD4 $^{+}$ CD8 $^{-}$ TCCs specific to Phl p 5 were previously established from the peripheral blood of three grass-allergic patients [25]. All TCLs and 15/34 TCCs tested reacted with either recombinant Phl p 5a or b or both. Table 1 shows the characteristics of the Phl p 5-specific TCCs that were further analysed in the present study.

The TCR usage for the Phl p 5-specific TCC was analysed by flow cytometry analysis (not shown). All TCCs expressed TCR $\alpha\beta$ molecules, while only one clone expressed V α 12.1 (AGC12), and none of the TCCs expressed V α 2. Four of the 13 TCCs (31%) tested, exclusively expressed the TCR V β 3.1 gene product compared with 5% TCR V β 3.1 $^{+}$ T cells in human peripheral blood, suggesting a biased TCR V β usage among Phl p 5-specific TCCs. The TCR V β element expressed by the remaining nine TCCs was not detected by the TCR V β -specific mAb panel used covering approximately 35% of the TCR repertoire observed on CD4 $^{+}$ T cells of the peripheral blood (ranging from 1 to 7% for each V β element) [27,28].

Epitope specificity of Phl p 5 isoallergen-specific TCCs and TCLs

The differential isoallergen recognition exhibited by the Phl p 5-specific TCCs investigated (Table 1) show that peptides or allergen fragments representing both Phl p 5a and 5b should be included to determine the epitope specificities of all the TCCs. To this aim the Phl p 5-specific TCLs and TCCs were stimulated with recombinant Phl p 5a peptide fragments and one synthetic peptide representing the entire Phl p 5a sequence, and six synthetic peptides representing three Phl p 5b sequence stretches with a high degree of sequence dissimilarities compared with Phl p 5a (Fig. 1).

T-cell lines The response of Phl p 5-specific T-cell lines

Table 1. Characteristics of Phl p 5-specific CD4⁺ TCCs

Clone	HLA restriction	Cross-reactivity	Isoallergen specificity	Phenotype
<i>Patient AGC</i>				
AGC12	HLA-DQ	Poa p 5/Lol p 5	Phl p 5a/Phl p 5b	TH0
AGC29	HLA-DQ	Poa p 5	Phl p 5b	TH2
AGC31	HLA-DQ	Poa p 5	Phl p 5b	TH0
AGC32	HLA-DQ	Poa p 5	Phl p 5b	TH2
AGC36	HLA-DQ	Poa p 5	Phl p 5b	TH2
AGC37	HLA-DQ	Poa p 5	Phl p 5b	TH1
<i>Patient MSC</i>				
MSC01	HLA-DQ	Poa p 5	Phl p 5a	TH2
MSC03	HLA-DR	Poa p 5/Lol p 5	Phl p 5a	TH0
MSC04	HLA-DR	Poa p 5/Lol p 5	Phl p 5b	TH0
MSC05	HLA-DR	Poa p 5	Phl p 5a/Phl p 5b	TH0
MSC12	HLA-DR	Poa p 5/Lol p 5	Phl p 5a	TH0
MSC15	HLA-DR	Poa p 5/Lol p 5	Phl p 5a/Phl p 5b	TH0
MSC17	HLA-DR	Poa p 5/Lol p 5	Phl p 5a	TH0
MSC19	HLA-DR	Poa p 5/Lol p 5	Phl p 5b	TH1
<i>Patient TL</i>				
G08TL	HLA-DR	Poa p 5/Lol p 5	Phl p 5a	TH0

The HLA restriction was determined in inhibition experiments using anti-HLA-DP, -DQ, or -DR mAbs. The cross-reactivity to the group 5 allergens of *Poa pratensis* and *Lolium perenne* as well as the recognition of recombinant Phl p 5 isoallergens was investigated by standard proliferation assays. Allergen-specific cytokine production was assayed in cell supernatants by ELISAs for IL4 and IFN γ . TH1: IL4/IFN γ < 0.1, TH0 0.1 < IL4/IFN γ < 10, and TH2 IL4/IFN γ > 10.

revealed at least three distinct Phl p 5a epitopes, which might be shared by Phl p 5b, and three distinct Phl p 5b T-cell epitopes in Phl p 5b amino acids (a.a.) 37–52, a.a. 50–65, and a.a. 225–251 not shared by the Phl p 5a isoallergen. Different combinations of epitopes were recognized by the TCLs from each of the patients. Two patients both recognized epitopes in the N-terminal and the C-terminal part of Phl p 5a, but responded to different Phl p 5b unique epitopes, and the third patient recognized several epitopes scattered throughout the Phl p 5a molecule and a single Phl p 5b unique epitope (Fig. 2).

T-cell clones T-cell epitopes in Phl p 5a a.a. 8–63, a.a. 120–140, and a.a. 189–256 and Phl p 5b a.a. 49–67 were recognized by the Phl p 5-specific TCCs isolated from the TCLs (Table 2). Three epitope stretches in Phl p 5a were recognized by Phl p 5a-specific TCCs as well as TCCs reacting to both Phl p 5a and 5b, indicating that several minimal T-cell epitopes were found within each of these sequence stretches (Fig. 3). The epitope specificity of all Phl p 5a-specific TCCs was determined, indicating that the minor sequence dissimilarities observed for the Phl p 5a peptide fragments compared with Phl p 5a did not influence

the T-cell activation. Interestingly, several Phl p 5b-specific TCCs did not respond to any of the Phl p 5b peptides indicating that additional Phl p 5b unique epitope(s) were recognized by these TCCs.

Surprisingly, TCC AGC12 was equally activated by sequence stretches within Phl p 5a a.a. 8–63 and a.a. 189–256. This was possibly caused by the presence of homologous sequence stretches within Phl p 5a (Fig. 3), rather than oligoclonality of the TCCs as AGC12 expressed V α 12.1 and V β 3.1 on 100% of the cells. Five other TCCs exhibited moderate proliferative responses, that were five- to tenfold lower than the epitope identified, to fragments of Phl p 5 representing sequences other than their main epitope specificity (Table 2), but none of the TCCs responded to all recombinant proteins or protein fragments excluding reactivity to the his-tag tail attached to the recombinant materials.

T-cell lines and T-cell clones In combination, the reactivity of the TCLs and TCCs reveal the existence of at least two distinct Phl p 5a unique epitopes, three epitopes shared by Phl p 5a and 5b and three Phl p 5b unique epitopes (Fig. 3). An additional T-cell epitope in Phl p 5a a.a. 132–189 possibly

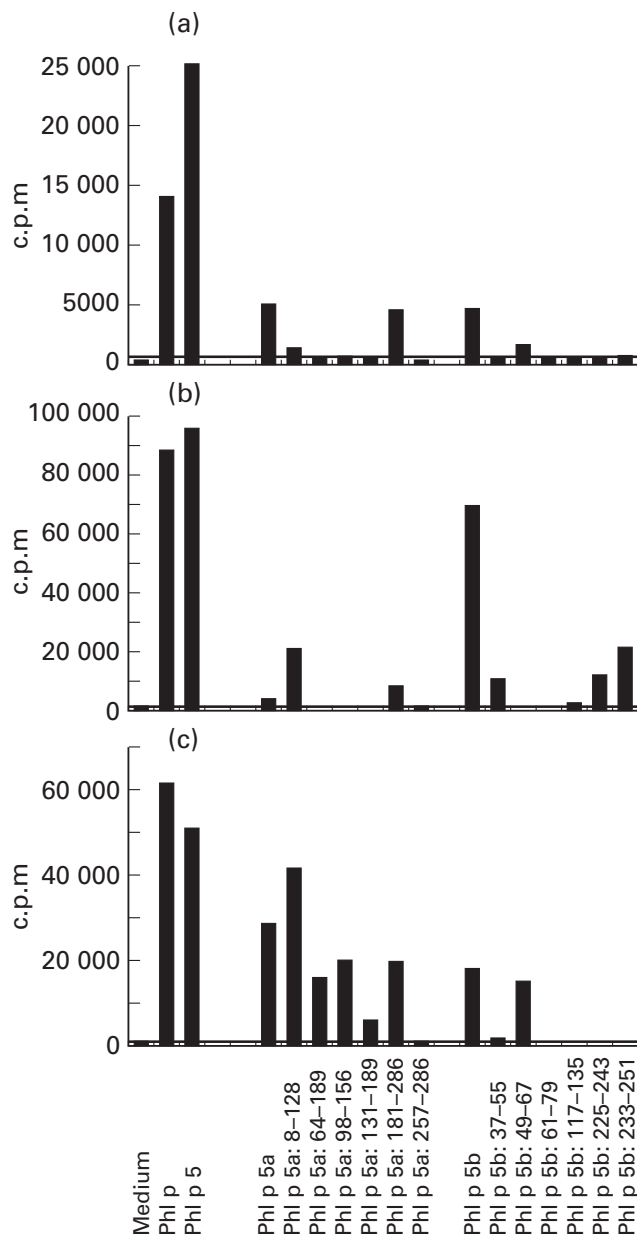


Fig. 2. Recognition of Phl p 5a and Phl p 5b peptides by Phl p 5-specific TCLs. T cells ($10^5/\text{mL}$) were stimulated with Phl p allergen extract ($20 \mu\text{g}/\text{mL}$), Phl p 5a ($10 \mu\text{g}/\text{mL}$), recombinant Phl p 5a or 5b ($5 \mu\text{g}/\text{mL}$), recombinant Phl p 5a peptides (diluted 1:40), synthetic peptides ($2 \mu\text{g}/\text{mL}$) or medium control in the presence of irradiated autologous PBMCs ($5 \times 10^5/\text{mL}$). Lines indicating $3 \times$ background c.p.m. are included to indicate the lower limit for positive responses. Comparable results were obtained in at least two separate experiments. Patients (a) TL, (b) AGC and (c) MSC.

shared by Phl p 5b was recognized by the MSC TCLs but not by any of the TCCs (Table 2, Fig. 2).

HLA restriction of Phl p 5 specific TCC

The HLA class II restriction of Phl p 5-specific TCCs determined by inhibition assays using mAb specific to HLA-DP, -DQ or -DR molecules were previously reported for the Phl p 5-specific TCCs (Table 1) [25]. To describe the fine HLA restriction specificity of the Phl p 5a- and Phl p 5b-specific TCCs, stimulation assays using HLA-typed PBMCs as antigen-presenting cells (APCs) were performed. Only HLA-DQ-B10602 restricted TCCs were identified from patient AGC (2/2), while one HLA-DQ-B10602 restricted, two HLA-DR-B11501/-B50101 restricted, and four HLA-DR-B10101 restricted TCCs from patient MSC were identified (Table 3). Two TCCs (MSC03 and MSC12) specific to epitopes within the same narrow sequence (Phl p 5a 8–63) were restricted by HLA-DR-B1-1501/-B5-0101 and HLA-DR-B1-0101, respectively, and even used different TCR V β elements (Table 4).

Discussion

In the present study, at least nine distinct T-cell epitopes of Phl p 5a and Phl p 5b isoallergens recognized by T cells from three grass-allergic patients were identified. Two of those epitopes were Phl p 5a unique, three were Phl p 5b unique and four epitopes were shared by Phl p 5a and Phl p 5b. The epitope recognition pattern differed considerably among patients. The HLA restriction of the TCCs was highly diverse, whereas an over-representation of TCR V β 3.1 $^+$ TCCs was found among Phl p 5-specific TCCs.

We recently characterized the highly heterogeneous Phl p 5-specific T cells of atopic patients and found that individual Phl p 5 isoallergens were differentially recognized by TCCs [25]. This indicated that a characterization of T-cell epitopes of this major allergen should be based on sequences representing several isoallergens/isoallergen groups, to ensure that the epitope specificity of all Phl p 5-specific TCCs could be identified. In this study only TCLs and TCCs specific to the known isoallergens Phl p 5a and Phl p 5b were included and tested with recombinant peptide fragments and synthetic peptides representing both these isoallergens.

The grass-allergic patients MSC, AGC and TL recognized seven, four and three distinct T-cell epitopes, respectively, and a total of at least nine distinct T-cell epitopes were recognized by the Phl p 5-specific T cells isolated from these three patients (Fig. 3). For patient MSC the specificity of eight TCCs was determined, but only two epitope stretches were recognized by more than one TCC, demonstrating the

Table 2. Epitope specificity for Phl p 5-specific TCCs

Clone	Isoallergen specificity	Epitope specificity	Cross-reactivity to nonoverlapping sequences
AGC12	Phl p 5a/Phl p 5b	a.a. 8–63 (26) & a.a. 189–256 (38)	–
MSC04	Phl p 5a/Phl p 5b	a.a. 8–63 (44)	–
MSC15	Phl p 5a/Phl p 5b	a.a. 120–140 (202)	a.a. 8–63 (25)
MSC01	Phl p 5a	a.a. 120–140 (102)	a.a. 8–63 (9)
MSC03	Phl p 5a	a.a. 8–63 (61)	a.a. 98–131 (13)
MSC12	Phl p 5a	a.a. 8–63 (84)	–
MSC17	Phl p 5a	a.a. 8–63 (225)	a.a. 98–131 (10)
G08TL	Phl p 5a	a.a. 8–63 (55)	a.a. 98–131 (12) & a.a. 189–256 (14)
MSC05	Phl p 5b	a.a. 49–67 (180)	–
MSC19	Phl p 5b	a.a. 49–67 (15)	–
AGC29	Phl p 5b	–	–
AGC31	Phl p 5b	–	–
AGC32	Phl p 5b	–	–
AGC36	Phl p 5b	–	–
AGC37	Phl p 5b	–	–

T-cells were stimulated as described in the legend of Fig. 2. The amino acid sequences recognized by Phl p 5-specific TCCs are shown in column three. Several Phl p 5a-specific TCCs exhibited additional but lower responses to nonoverlapping sequences within Phl p 5a as shown in column four. Numbers in brackets indicate SI values (c.p.m. with Ag/background c.p.m.). Background values (TCCs cultured in medium) were in all cases below 500 c.p.m. All proliferative responses above 5 SI are shown.

highly diverse epitope specificity of Phl p 5-specific TCCs of individual patients (Table 2). Phl p 5a 8–63 and 189–257 were recognized by T cells of all three patients. However, epitopes in Phl p 5a 120–180 were also important for patient MSC, since the TCL and two of the eight TCCs of this

patient recognized this region. Furthermore, patients TL and MSC exclusively recognized Phl p 5b a.a. 49–67, while Phl p 5-specific T cells of patient AGC responded to two distinct epitopes in other regions of the Phl p 5b molecule (Phl p 5b a.a. 37–55 and a.a. 225–251).

Phl p 5a	1	ADLGYGEATPAAPAAGYTPATPAAPAGADAAGKATTEEOKLIEKINAGFKAALAGAGVQP-ADKYRTFVATFGPASNKAF	
Phl p 5b	1	..A..A.....D..V...V.A.ASV.A...fk...E.A.TSs.KA.A	
Phl p 5a	81	AEGLSGEPKGAESSKAALTSKLDAAKYKLAYKTAEGATPEAKYDAYVATLSEALRIAGTLEHVAVKPAEE--VKVIP	
Phl p 5b	67	.kA-----PG.VP.....Sv...A.V.....f.sv..s.t....v...A.....VT..PGmAK..	

Phl p 5a	159	AGELQVIEKVDAAFKVAATAANAAPANDKFTVFEEAFNDEIKASTGGAYESYKFIPALEAAVKQAYAATVATAPEVKYTV	
Phl p 5b	135	i.d.i.....AT...d.....KA..E.....dt..C..S.....A..q...A.	
Phl p 5a	239	FETALKKAITAMSEAQAAPKP---AAAATATATAAVGAATGAATAATGGYKV*	286
Phl p 5b	215	..A..T....V..Vsqa.TGA.TV.AGA..T.A...s....V.A.....*	266

Fig. 3. Amino acid sequences for Phl p 5a and Phl p 5b isoallergens. Identical a.a. are indicated by dots, gaps are represented by dashes. Boxed regions contain T-cell epitopes recognized by Phl p 5-specific TCC or TCL from grass allergic patients. Double lined boxes indicate that Phl p 5a-specific and TCC reacting to both isoallergens recognized these epitope stretches, suggesting the existence of several minimal T-cell epitopes within each of these sequences. *indicates sequence stretches in Phl p 5a with more than 40% identity possibly explaining the equal reactivity of TCC AGC12 to Phl p 5a a.a. 8–63 and a.a. 189–256.

Interestingly, several TCCs recognizing Phl p 5a responded to two distinct, nonoverlapping Phl p 5a fragments (Table 2). This intra-Phl p 5a cross-reactivity may reflect the high degree of sequence homology found within Phl p 5a (Fig. 3). An alternative explanation is that the TCCs

investigated were oligoclonal, but the fact that the TCCs exhibiting intramolecular cross-reactivity were restricted by single HLA molecules (Table 3) makes this possibility highly unlikely. In addition, AGC12 and MSC03, two TCCs that displayed intramolecular cross-reactivity, used

Table 3. HLA restriction specificity of HLA-DQ- and HLA-DR-restricted Phl p 5-specific TCCs

(a)						
HLA-DQ of donor		AGC12	AGC37	HLA-DQ of donor		MSC01
Autologous				Autologous		
DQA10102	Phl p 5	20.0	26.0	DQA10101, 0102	Phl p 5	2.0
DQB10602, 0604	Medium	1.3	0.3	DQB10501, 0602	Medium	0.1
HLA matched				HLA matched		
DQA10102	Phl p 5	21.5	20.0	DQA10102	Phl p 5	11.5
DQB10602	Medium	1.5	0.2	DQB10602, 0604	Medium	0.1
DQA10102	Phl p 5	9.0	4.0	DQA10102	Phl p 5	0.3
DQB10604	Medium	7.0	0.6	DQB10604	Medium	0.6
HLA miss match				HLA miss match		
DQA10103, 0301	Phl p 5	6.5	2.5	DQA10101, 0302	Phl p 5	0.1
DQB10302, 0603	Medium	2.5	0.1	DQB10501, 0302	Medium	0.1
				HLA miss match		
				DQA10103, 0301	Phl p 5	0.1
				DQB10302, 0603	Medium	0.1
HLA restriction		DQB1 0602	DQB1 0602	HLA restriction		DQB1 0602

(b)							
HLA-DR of donor		MSC03	MSC04	MSC05	MSC12	MSC15	MSC17
Autologous							
DRB10101, 1501	Phl p 5	12.0	30.0	6.3	3.0	12.0	14.0
DRB50101	Medium	0.1	0.1	0.3	0.1	0.2	2.5
HLA matched							
DRB11501	Phl p 5	21.0	0.7	0.6	1.4	0.8	36.0
DRB30103	Medium	0.6	0.2	0.3	2.1	0.3	4.5
HLA miss match							
DRB50101							
DRB10101,0401	Phl p 5	2.7	40.0	12.5	4.5	43.5	2.3
DRB40301	Medium	0.1	0.1	0.1	0.1	0.2	3.2
HLA miss match							
DRB11302,0404	Phl p 5	0.4	0.1	0.3	0.2	0.2	2.7
DRB30101	Medium	0.1	0.1	0.2	0.1	0.3	2.3
HLA restriction							
		DRB1 1501/ DRB5 0101	DRB1 0101	DRB1 0101	DRB1 0101	DRB1 0101	DRB1 1501/ DRB5 0101

Phl p 5 specific and background proliferation (c.p.m. $\times 10^3$) for HLA DQ restricted (a) or HLA DR restricted (b) TCC (10^5 /mL) stimulated with Phl p 5 ($10 \mu\text{g}/\text{mL}$) in the presence of irradiated HLA typed PBMC (5×10^5 /mL). Positive stimulations in bold.

Table 4. HLA restriction, TCR usage and specificity for Phl p 5-specific TCCs

Clone	HLA restriction	TCR		Specificity
		V α	V β	

<i>Patient AGC</i>				
AGC12	DQB1*0602	V α 12.1	V β 3.1	Phl p 5a: a.a. 8–63 and a.a. 189–256/Phl p 5b
AGC29	ND	–	V β 3.1	Phl p 5b
AGC31	ND	–	–	Phl p 5b
AGC32	ND	–	–	Phl p 5b
AGC36	ND	–	V β 3.1	Phl p 5b
AGC37	DQB1*0602	–	–	Phl p 5b
<i>Patient MSC</i>				
MSC01	DQB1*0602	–	–	Phl p 5a: a.a. 120–140
MSC03	DRB1*1501/B3*0101	–	V β 3.1	Phl p 5a: a.a. 8–63
MSC04	DRB1*0101	ND	ND	Phl p 5a: a.a. 8–63/Phl p 5b
MSC05	DRB1*0101	ND	ND	Phl p 5b: a.a. 49–67
MSC12	DRB1*0101	–	–	Phl p 5a: a.a. 8–63
MSC15	DRB1*0101	–	–	Phl p 5a: a.a. 120–140/Phl p 5b
MSC17	DRB1*1501/B3*0101	–	–	Phl p 5a: a.a. 8–63
MSC19	ND	–	–	Phl p 5b: a.a. 49–67
<i>Patient TL</i>				
G08TL	ND	ND	ND	Phl p 5a: a.a. 8–63

The table summarizes the results presented. HLA restriction was determined in proliferation assays with HLA typed APC, TCR usage was detected by flow cytometry, and epitope specificity was determined in proliferation assays with recombinant peptide-fragments and synthetic peptides representing the sequences of Phl p 5a and Phl p 5b. ND: not determined, –: no binding of mAb in panel.

a single TCR V β gene product, which further points to the clonality of these TCCs (Table 4). The intra-Phl p 5a cross-reactivity patterns of Phl p 5a-specific TCCs show that all the Phl p 5a-specific TCCs investigated were activated to some extent when stimulated with Phl p 5a 8–63. This could indicate that this sequence stretch might function as an immunodominant T-cell epitope of Phl p 5a. Unfortunately, the existence of at least three distinct Phl p 5b epitopes in the N-terminal and C-terminal part of the molecule and an unknown number of Phl p 5 epitopes on the additional isoallergens recognized by Phl p 5-specific T cells [25] reduces the applicability of such a possible immunodominant T-cell epitope region. Peptide treatment may downregulate the T-cell response to epitopes in other parts of the full-length molecule through the induction of linked suppression as demonstrated in mouse model systems [29]. However, in contrast with the situation in humans, the T-cell reactivity induced in these mice is almost exclusively responding to clearly immunodominant T-cell epitopes among the very limited number of epitopes recognized. In addition, the downregulation of the T-cell response to the full-length proteins by peptide treatment seems to dependent on the presentation of the small (10–15 a.a.) peptides by non-professional APCs. This is unlikely to

happen if long (60–100 a.a.) peptides are used for treatment and may explain the limited success of such preparations for the treatment of allergic disease [30].

Specific immunotherapy (SIT) modulates the allergen-specific immune response by exposing the immune system to increasing doses of the allergen. After successful SIT a shift in the cytokine production profile of allergen-specific T cells from TH2 cytokines to TH1 cytokines is observed [31–34], indicating that T lymphocytes may be the principal target cells for this specific allergy treatment. The finding that allergen-specific T-cell responses *in vitro* can be modulated by treatment with allergenic peptides containing major or immunodominant T-cell epitopes [29,35] suggested that the T-cell response of allergic patients might be modulated by treatment with peptide preparations. However, the high number of T-cell epitopes and the variable epitope recognition pattern from patient to patient found for Phl p 5 in the present study (Fig. 2, Table 2) and previously described for grass group 1 allergens [15,36], imply that full-length allergens are necessary to target the existing T-cell response of grass-allergic patients. For Phl p 5 the T-cell-activating capacity of preparations of recombinant allergens intended for SIT should be validated in a large patient group since the recognition of individual T-cell epitopes differs

considerably between patients. In addition the need for large patient groups is further stressed by the fact that the isoallergenic variation of Phl p 5 markedly influences T-cell recognition and that approximately 50% of Phl p 5-specific TCCs recognize Phl p 5 isoforms other than the Phl p 5a and 5b isoallergens previously tested [25]. Interestingly, a low IgE-binding Bet v 1 isoallergen [37] and genetically modified recombinant Der p 2 with lowered IgE-binding capacity [38] have been suggested for SIT to reduce the risk of IgE-mediated side-effects of this treatment. If such low IgE-binding allergen preparations retain a T-cell-activating ability comparable with that of the natural allergens, they may bring further improvement for new treatment protocols for specific therapy.

Thirty-one percent (4/13) of the Phl p 5-specific TCCs investigated expressed TCR V β 3.1, while on average approximately 5% of human peripheral blood T cells have been described to express this V β element [27,28]. This indicates that an over-representation of V β 3.1⁺ T cells among Phl p 5-specific T cells from grass-allergic patients appears to exist. Similarly, Wedderburn *et al.* [39] described an over-representation of V β 3.1 TCCs specific to major house dust mite allergen Der p 1. A restricted TCR V α 13 usage for Phl p 5-specific TCCs as previously described for Poa p 5-specific TCCs [18] could not be excluded on basis of the present results. However, TCR V α 12.3 usage was demonstrated for AGC12 (Table 4), indicating that TCR V α gene products other than V α 13 are involved in the recognition of grass group 5 allergens. A diverse TCR V β usage has been reported for several allergens [8,9,40–43] including Der p 1 [44] and the TCR gene products involved in the T-cell recognition of Bet v 1 were even shown to differ among TCCs with identical epitope specificity [41–43]. The panel of TCR V β -specific mAbs used in the present study covers approximately 35% of the TCR V β repertoire observed in the peripheral blood [27,28], implying that the TCR V β usage for the remaining nine TCCs may be highly diverse. This suggests that the slight bias towards usage of TCR V β 3.1 by Phl p 5-specific T cells of allergic patients shown here does not justify TCR-targeted intervention for treatment of grass allergy.

HLA-DRB1*1501/B5*0101, -DRB1*0101 and -DQB1*0602 were involved in the T-cell response to Phl p 5 (Table 3) and all three HLA restriction elements were involved in the response of patient MSC. In addition, two TCCs (MSC03 and MSC12) recognizing epitopes within the same limited Phl p 5 sequence stretch (Phl p 5a 8–63) were restricted by HLA-DR-B1*1501/B5*0101 and HLA-DR-DRB1*0101, respectively. This clearly demonstrates the diversity of HLA-restriction for Phl p 5-specific T cells of atopic patients, within individual patients as well as for the presentation of narrow amino acid sequences. A comparable diversity with respect to HLA restriction has

been demonstrated for the recognition of T-cell epitopes of several major allergens as could be expected on basis of the high number of epitopes recognized in most allergens [8].

In conclusion, the diversity of the Phl p 5-specific T-cell response with respect to HLA restriction and epitope recognition underscores the diversity of the T-cell response to this major grass allergen (Table 4). This indicates that immunotherapy with allergenic peptides is not feasible for treatment of grass allergy. If the present results are confirmed in studies on larger patient groups new treatment strategies for grass allergen immunotherapy may instead be based on mixtures of carefully selected full-length rPhl p 5 isoallergens with a T-cell-activating capacity comparable with that of natural Phl p 5.

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