

Mapping of T-cell epitopes of Phl p 5: evidence for crossreacting and non-crossreacting T-cell epitopes within Phl p 5 isoallergens

W.-D. MÜLLER*, T. KARAMFILOV*, H. KAHLERT†, H. T. STÜWE†, B. FAHLBUSCH*, O. CROMWELL†, H. FIEBIG† and L. JÄGER*

*Institute of Clinical Immunology, Friedrich Schiller University, Jena and †Allergopharma J. Ganzer KG, Reinbek, Germany

Abstract

Background Group 5 allergens represent major grass pollen allergens because of their high sensitization indices. The identification of T-cell epitopes of these allergens is a prerequisite for the design of immunotherapeutic strategies based on peptide vaccination or modified allergens with conserved T-cell epitopes.

Objective This study was undertaken to determine T-cell epitopes on Phl p 5 major pollen allergen of timothy grass (*Phleum pratense*).

Methods T-cell lines (TCLs) and T-cell clones (TCCs), specific to Phl p 5, were established from the peripheral blood of 18 patients allergic to grass pollen. All TCCs were mapped for epitope specificities using 178 overlapping dodecapeptides representing the primary structures of two isoforms of Phl p 5 (Phl p 5a and Phl p 5b). Phenotype and cytokine production profiles of TCCs were tested. Selected TCCs were analysed for HLA class II restriction.

Results A total of 82 TCCs were isolated. All TCCs displayed the helper cell (TH) phenotype. Their reactivity with two recombinant expressed isoforms of Phl p 5a and Phl p 5b was heterogeneous. The epitope specificity of the TCCs was then revealed. Nineteen T-cell epitopes could be identified on Phl p 5. Eighty-one percent of mapped TCCs recognized three T-cell reactive regions on the Phl p 5 allergen. Some TCCs were reactive with isoepitopes presenting on Phl p 5a as well as Phl p 5b. Allergen-specific stimulation induced a TH₀-like type of cytokine production in 25 of 50 TCCs. Almost all TCCs secreted high concentrations of interleukin-13.

Conclusion Phl p 5, a major grass pollen allergen, contains several T-cell epitopes. Some epitope regions were recognized by several patients. Epitope recognition pattern could not be correlated with special HLA class II haplotypes. T-cell stimulating isoepitopes were found at corresponding regions of Phl p 5a and Phl p 5b isoforms.

Keywords: Type I allergy, Phl p 5, recombinant Phl p 5, T-cell clone, T-cell epitope

Clinical and Experimental Allergy, Vol. 28, pp. 1538–1548. Submitted 3 February 1998; revised 3 May 1998; accepted 26 May 1998.

Introduction

Common environmental allergens such as grass pollen are the major causes of IgE (Type 1) -mediated allergic disease.

Various components of pollen grains have been shown to be responsible for this allergic immune response [1]. In grasses, six groups of pollen allergens are well established. The group 1 and 5 allergens are represented in almost all grasses studied hitherto [2], and the sensitization indices of group 1 and 5 allergens indicate that they are of particular importance [3]. More than 95% of grass pollen-allergic individuals display IgE-reactivity to group 1 allergens

Correspondence: Dr W.-D. Müller, Institute of Clinical Immunology, Friedrich Schiller University, Am Johannisfriedhof 3, 07743 JENA, Germany.

which represent glycoproteins of ≈ 30 kDa that occur in most grass species [4]. Group 5 allergens, though bound by fewer patients (80% of patients with grass pollen allergy), are of particular importance because of their extremely high IgE-binding capacity [5].

T cells play a key role in the induction, maintenance or down-regulation of allergen-specific IgE synthesis. Recently, the use of molecular biological techniques has provided the means to map complete amino acid sequences of most of the important major grass pollen allergens, including group 5 allergens. Timothy grass pollen group 5 allergens can be distinguished by molecular weight into groups 5a and 5b [6,7]. Complete cDNA and deduced amino acid sequences of Phl p 5a and Phl p 5b have been published recently [8–10]. Furthermore, various isoforms of Phl p 5a as well as of Phl p 5b have been described [11–13]. Therefore the identification of T-cell epitopes on group 5 grass pollen allergens has become possible. However, only a few studies have investigated the response to the group 5 allergens at the specific T-cell level, and reliable data concerning defined T-cell epitopes have been provided by only two groups [14,15]. In the present study it was established that T-cell lines (TCLs) from individuals allergic to grass pollen by selection with a natural group 5 allergen of timothy grass pollen. Natural Phl p 5 (nPhl p 5) was isolated from a timothy grass pollen extract by immunoaffinity chromatography using a specific monoclo-

nal antibody [16,17]. After cloning the TCL, the reactivities of the isolated TCC were tested with one recombinant (r) isoform of each of Phl p 5a and Phl p 5b. Overlapping peptides synthesized according to the amino acid sequences of both isoforms (rPhl p 5a and rPhl p 5b) were used to identify T-cell epitopes. The identification of T-cell epitopes of group 5 allergens will contribute to the design of immunotherapeutic strategies based on peptide vaccination, or modified allergens.

Materials and methods

Patients

Peripheral blood was collected from 18 patients allergic to grass pollen. Type 1 allergy to grass pollen was defined by typical anamnesis, positive skin-prick test and timothy grass-specific IgE, as measured by radioallergosorbent test (RAST). Furthermore, plasma samples of these patients were tested for positive IgE reactivity with solid phase bound nPhl p 5 allergens in a direct enzyme-linked immunosorbent assay (ELISA; data not shown). Five of the 18 blood donors (DW, CB, GE, CBH and USR) had undergone allergen-specific immunotherapy before establishing TCCs. The clinical data of the 18 timothy grass pollen-sensitive subjects are shown in Table 1.

Table 1. Clinical data and timothy grass pollen-specific IgE of blood donors

Patient	Age/Sex	Atopic Disease	RAST Classification (timothy grass-specific IgE) ^c	HLA-DR Haplotype
DW	37/M	AR ^b	3.0	15
CB	27/F	A ^a ; AR	> 4.0	4,17
JR	27/M	A; AR	> 4.0	1
KS	25/F	AR	3.8	11,15; W51,W52
KSE	23/F	A; AR	3.4	10,13; W52
GE	26/M	AR	3.0	4,7; W53
AS	21/M	AR	3.2	7,13; W52,W53
UZ	26/M	AR	> 4.0	11,15; W51,W52
UD	26/M	A; AR	3.0	11,17; W52
CBH	23/F	AR	> 4.0	7,11; W52,W53
UZH	27/F	A; AR	> 4.0	4,8; W53
USR	24/M	AR	3.1	7,13; W52, W53
JMD	26/M	AR	3.0	11,17; W52
MF	28/M	AR	> 4.0	4,10; W53
AH	29/M	AR	> 4.0	4,7; W53
JS	28/M	AR	> 4.0	n.t.
TB	30/M	AR	> 4.0	n.t.
MD	42/F	A; AR	> 4.0	n.t.

^aA, asthma; ^bAR, allergic rhinitis; ^c, Phadebas RAST (Pharmacia diagnostics; Uppsala, Sweden).

Phl p 5a:

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ADLGYPATPAAPAAGYTPATPAAPAGADAAGKATTEEQKLEKINAGFKAALAGAGVQPADKYRT
1      10      20      30      40      50      60
FVATFGPASNKAFAEGLSGEPKGAAESSKAALTSKLDAAAYKLAYKTAEGATPEAKYDAYVATLSEA
70      80      90      100     110     120     130
LRIIAGTLEHVAVKPAAEVVKVIPAGELQVIEKVDAAFKVAATAANAAPANDKFTVFEEAFNDEIKA
140     150     160     170     180     190     200
STGGAYESYKFIPALEAAVKQAYAATVATAPEVKYTVFETALKKAITAMSEAQKAAKFAAAATATA
210     220     230     240     250     260
TAAVGAATGAATAATGGYKV
270     280     286

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Phl p 5b: Essential T-cell reactive regions on the Phl p 5b allergen (see also Table 3) are underlined

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ADAGYAPATPAAAGAAAGKATTEEQKLIEDINVGFKA AVAAA SVPAADKFKTFEAAFTSSSKAAAAPGL
1      10      20      30      40      50      60      70
VPKLDAAYSVAYKAAVGATPEAKFDSFVASLTEALRVIAGALEVHAVKPVTEEPGMAKIPAGELQIIDKIDAAF
80      90      100     110     120     130     140
KVAATAAATAPADDKFTVFEEAFNKAIKESTGGAYD TYKCIPSLEAAVKQAYAATVAAAPOVKYAVFEAAL
150     160     170     180     190     200     210
TKAITAMSEVQKVSQPATGAATVAAGAATTAAGAASGAATVAAGGYKV
220     230     240     250     260     265

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Fig. 1. Sequences of the Phl p 5a allergen and the Phl p 5b allergen. Numbers indicate amino acid residues (international one-letter code). A total of 178 dodecapeptides were synthesized according to the Phl p 5a and Phl p 5b sequences.

Allergens

Lyophilized pollen extract of *Phleum pratense* (timothy) was provided by Allergopharma J. Ganzer KG (Reinbek, Germany). Natural group 5 allergens (nPhl p 5) from timothy grass pollen extract were isolated by immunoaffinity chromatography using the monoclonal antibody 1D11 [18,19] as described elsewhere [16]. Two recombinant Phl p 5 isoallergens (Iso A3 and Iso B5 according to 13) were kindly provided from Dr A. Bufer (Borstel, Germany). All Phl p 5 allergens were dissolved in culture medium and sterile filtered prior to use in cell culture.

Peptides

A total of 178 dodecapeptides (offset by three residues and overlapping by nine residues) were synthesized: 92 dodecapeptides according to the Phl p 5a sequence, and 86 dodecapeptides according to the Phl p 5b sequence (see Fig. 1). The peptides were synthesized using a commercial, Multi Pin™ DKP-Peptide synthesis Kit (CHIRON Mimotopes Peptide Systems, Clayton, Vic., Australia). Epitope mapping of TCCs was performed for each of these peptides

at a concentration of 1 µg/mL. Four selected peptides representing two (A and B) corresponding isoeptopes of Phl p 5a and Phl p 5b were used for crossreactivity studies (bold letters indicate amino acid exchanges between the corresponding peptide):

Epitope A:

... YKCIPSLEAAVK ... (Phl p 5b; AAs 184–195)
... YKFIPALEAAVK ... (Phl p 5a; AAs 209–220)

Epitope B:

... LQIIDKIDAAFK ... (Phl p 5b; AAs 136–147)
... LQVIEKVDAAFK ... (Phl p 5a; AAs 161–172)

Isolation of Phl p 5 reactive TCCs

Phl p 5 reactive TCCs were isolated according to a method recently described by the authors [20]. Briefly, 2×10^6 peripheral blood mononuclear cells (PBMC) were stimulated in 24-well, flat-bottomed plates (NUNC, Roskilde, Denmark) with 10 µg/mL purified Phl p 5 for 7 days in Ultra

Table 2. Epitope specificities and proliferative responses of Phl p 5 reactive TCCs

Epitope AAs on			Proliferative responses (c.p.m.) ^a			
TCC	rPhl p 5a	rPhl p 5b	no Ag ^b	nPhl p 5	rPhl p 5a	rPhl p 5b
DW 8	161–172	136–150	25	<u>2250</u>	75	<u>2780</u>
DW 14	–	196–207	15	<u>1800</u>	<u>1720</u>	<u>1290</u>
DW 16	209–220	181–195	35	<u>390</u>	280	250
DW 23	209–220	181–195	40	<u>1840</u>	<u>1920</u>	<u>760</u>
DW 25	209–220	184–195	90	<u>7110</u>	<u>5310</u>	<u>2610</u>
DW 28	209–220	184–195	50	<u>700</u>	<u>1400</u>	<u>500</u>
CBH 1	n.t.	211–225	15	<u>1200</u>	30	<u>180</u>
CBH 2	–	–	40	<u>480</u>	45	120
CBH 7	–	–	70	<u>1620</u>	140	280
CBH 10	n.t.	211–222	60	<u>5400</u>	70	<u>1440</u>
JR 6	161–172	136–150	40	<u>2800</u>	<u>1760</u>	160
JR 7	161–172	136–150	20	<u>1240</u>	<u>400</u>	<u>420</u>
JR 9	209–220	181–195	35	<u>1680</u>	<u>420</u>	<u>1500</u>
JR 10	–	19–30	90	<u>2250</u>	180	<u>1170</u>
JR 11	n.t.	49–60	100	<u>3100</u>	n.t.	n.t.
JR 13	209–220	181–195	60	<u>3240</u>	<u>2460</u>	<u>3960</u>
JR 15	209–220	181–195	50	<u>2100</u>	<u>950</u>	<u>1650</u>
JR 19	161–172	136–147	85	<u>2210</u>	340	<u>935</u>
JR 24	–	97–111	105	<u>1780</u>	n.t.	n.t.
JR 25	209–220	181–195	120	<u>2160</u>	840	<u>1440</u>
JR 27	209–220	184–195	110	<u>1100</u>	880	<u>660</u>
KS 1	209–220	181–195	55	<u>3300</u>	110	<u>1650</u>
KS 2	209–220	181–195	140	<u>1400</u>	150	<u>430</u>
KS 3	209–220	181–195	95	<u>1140</u>	100	<u>1050</u>
KS 4	209–220	181–195	80	<u>1120</u>	95	<u>320</u>
KS 5	209–220	181–195	75	<u>1130</u>	90	<u>1280</u>
KSE 9	46–57	–	40	<u>1760</u>	50	90
KSE 18	–	43–54	50	<u>1500</u>	110	<u>1050</u>
KSE 19	–	–	50	<u>2650</u>	200	<u>2300</u>
KSE 21	–	–	60	<u>960</u>	75	90
KSE 23	–	–	25	<u>1000</u>	35	20
KSE 29	46–57	–	35	<u>1015</u>	80	<u>630</u>
KSE 31	–	–	90	<u>950</u>	80	<u>720</u>
KSE 32	–	–	65	<u>1300</u>	140	<u>1430</u>
KSE 34	–	–	55	<u>2750</u>	160	<u>110</u>
UD 2	–	–	75	<u>1200</u>	n.t.	80
UD 5	–	–	70	<u>1610</u>	n.t.	100
UD 6	n.t.	112–123	15	<u>2160</u>	n.t.	40
1GE 3	–	–	100	<u>1200</u>	<u>1400</u>	850
GE 4	–	136–150	70	<u>1960</u>	150	90
GE 7	–	136–150	60	<u>2340</u>	65	140
GE 9	–	–	110	<u>1110</u>	<u>2310</u>	240
GE 11	–	–	120	<u>1560</u>	<u>370</u>	960
GE 12	–	37–48	75	<u>3080</u>	95	<u>2850</u>

Table 2. Continued

Epitope AAs on			Proliferative responses (c.p.m.) ^a			
TCC	rPhl p 5a	rPhl p 5b	no Ag ^b	nPhl p 5	rPhl p 5a	rPhl p 5b
AS 1	–	–	40	<u>1280</u>	50	60
AS 2	–	–	60	<u>2280</u>	250	195
AS 3	n.t.	163–174	90	<u>1080</u>	105	<u>3240</u>
AS 4	209–220	181–195	55	<u>1210</u>	<u>2150</u>	<u>120</u>
AS 5	209–220	181–195	70	<u>1190</u>	<u>1820</u>	150
AS 6	–	–	80	<u>1120</u>	100	175
UZH 1	–	–	110	<u>1210</u>	140	230
UZH 2	–	136–150	95	<u>970</u>	210	<u>1330</u>
UZH 6	232–243	–	40	<u>3200</u>	<u>3600</u>	65
UZ 6	232–243	–	25	<u>700</u>	<u>400</u>	60
UZ 8	–	–	20	<u>1800</u>	55	30
UZ 17	–	–	80	<u>880</u>	100	175
UZ 22	–	–	60	<u>1800</u>	150	165
UZ 24	161–172	–	100	<u>2200</u>	<u>1900</u>	<u>2800</u>
UZ 25	–	100–111	90	<u>3240</u>	360	<u>3330</u>
CB 1	n.t.	190–204	60	<u>1020</u>	130	<u>1140</u>
CB 2	–	181–195	40	<u>1000</u>	45	<u>1080</u>
CB 7	n.t.	25–36	40	<u>1040</u>	60	<u>1360</u>
CB 10	–	181–195	30	<u>1140</u>	75	<u>1050</u>
CB 14	–	181–195	35	<u>1400</u>	90	<u>1330</u>
USR 6	53–64	–	40	<u>1120</u>	<u>1520</u>	50
USR 8	–	–	15	<u>1580</u>	30	25
USR 9	–	–	20	<u>1560</u>	35	35
USR 11	–	–	15	<u>1290</u>	<u>990</u>	120
USR 12	–	–	90	<u>1080</u>	190	<u>1350</u>
MF 11	n.t.	184–195	60	<u>1140</u>	n.t.	<u>720</u>
JMD 3	n.t.	133–144	70	<u>1750</u>	n.t.	<u>1190</u>
AH 19	n.t.	16–27	25	<u>1680</u>	n.t.	<u>1130</u>
AH 26	n.t.	139–150	40	<u>1760</u>	n.t.	<u>1280</u>
JS 1	n.t.	31–42	100	<u>1500</u>	120	<u>1400</u>
TB 1	n.t.	196–207	90	<u>1350</u>	<u>1170</u>	<u>1080</u>
TB 2	n.t.	187–198	80	<u>1280</u>	90	<u>880</u>
MD 1	n.t.	184–195	110	<u>1320</u>	<u>1540</u>	<u>1980</u>
MD 2	n.t.	184–195	120	<u>1800</u>	<u>1560</u>	<u>2400</u>
MD 3	n.t.	184–195	60	<u>960</u>	<u>900</u>	<u>1020</u>
MD 4	n.t.	193–204	90	<u>1080</u>	360	810
MD 5	n.t.	163–174	80	<u>1200</u>	90	570
MD 6	n.t.	49–63	100	<u>1200</u>	120	840

a: Stimulation indices greater than 10 are underlined. b: c.p.m. obtained in cultures with TCCs plus irradiated autologous PBMC without antigen. (–, no identified epitope; n.t., not tested).

Culture medium (BioWhittaker, Walkersville, WV, USA) supplemented with 2 mmol/L L-glutamine (Sigma, St Louis, MO, USA) and 1% v/v antibiotic-antimycotic solution (Sigma). After a further stimulation period of 5 days in the presence of interleukin-2 (IL-2; 15 IU/mL; Pharma Biotechnology, Hannover, Germany), T-cell blasts were enriched on density gradients (Lymphoprep, Nycomed, Oslo, Norway). The Phl p 5 specificity of T-cell lines (TCLs) was assessed by a tritiated thymidine (^3H -TdR; Amersham, UK) incorporation assay as described under proliferation assays. TCLs were cloned by limiting dilution (0.3 cells/well, final volume 0.2 mL) in 96-well round-bottomed plates (NUNC) in the presence of irradiated (40 Gy) allogeneic PBMC (5×10^4 /well) as feeder cells, phytohaemagglutinin (PHA, 1 $\mu\text{g}/\text{mL}$; Biochrom, Berlin, Germany) and IL-2 (25 IU/mL). After 12–14 days, microcultures were fed with fresh irradiated allogeneic PBMC, PHA and IL-2. Furthermore, fresh IL-2 (25 IU/mL) was added at 4- to 5-day intervals. Before performing Phl p 5-specific proliferation assays, clones were settled for at least 10 days after the last addition of irradiated allogeneic PBMC. Selected Phl p 5-reactive TCCs were expanded in 24-well plates by restimulation with PHA (2.5 $\mu\text{g}/\text{mL}$), irradiated allogeneic feeder cells (5×10^5 /well) and IL-2 (50 IU/mL).

Proliferation assays

The specificity of TCL/TCC was assessed as follows: T-cell blasts (2×10^4 /well) were incubated in triplicate cultures in 96-well round-bottomed plates (final volume 0.2 mL) in the presence of irradiated autologous PBMC (5×10^4 /well) as antigen-presenting cells (APC), nPhl p 5, rPhl p 5a or rPhl p 5b (in each case 10 $\mu\text{g}/\text{mL}$). The unrelated antigen tetanus toxoid (10 $\mu\text{g}/\text{mL}$; Behring, Marburg, Germany) was used as a control for specificity. After 56 h ^3H -TdR (1 $\mu\text{Ci}/\text{well}$) was added for 16 h. Incorporated radioactivity was measured by a Matrix 96 beta counter (Canberra Packard, Meriden, CT, USA). The results are expressed as arithmetic mean counts per minute (c.p.m.) for triplicate cultures. Responses were considered positive when the stimulation index (i.e. the ratio between c.p.m. obtained in cultures with TCC plus irradiated autologous PBMC plus nPhl p 5 and the same cultures without nPhl p 5) was greater than 10. In each case data from representative experiments are shown.

Proliferation inhibition experiments

For testing of HLA class II restriction of selected TCC, monoclonal antibodies against HLA class II determinants (anti-HLA-DR, anti-HLA-DQ, anti-HLA-DP; Becton Dickinson, San Jose, TX, USA) were added (20 $\mu\text{g}/\text{mL}$) to nPhl p 5-driven T-cell proliferation. Inhibition of more than 50% of allergen-dependent proliferation was considered as positive.

Phenotype analysis of TCC

Analysis of TCC for expression of cell surface markers CD3, CD4, CD8 and TCR α/β was performed employing flow cytometry using appropriate fluorescein isothiocyanate-labelled monoclonal antibodies (Becton Dickinson) and FACScan (Becton Dickinson).

Phl p 5 stimulation of TCC for cytokine production

The Phl p 5 stimulated cytokine production (IL-4, γ -IFN, IL-13) was quantified for 50 TCCs with known epitope specificity. All three cytokines were measured by commercial ELISA kits (IL-4: R/D Systems, Minneapolis, MN, USA; γ -IFN: Laboserv, Gießen, Germany; IL-13: Bio Source International, Camarillo, CA, USA). Per well, 1×10^6 TCCs were added to 1×10^6 irradiated autologous PBMC in the absence or presence of 10 $\mu\text{g}/\text{mL}$ nPhl p 5 in 24-well flat-bottomed plates (final volume 1 mL). After incubation for 36 h, the cells were centrifuged, and culture supernatants were collected and stored in aliquots at -20°C until testing. Cultures containing either APC or TCC plus APC in the absence of allergen were used as negative controls.

Results

A total of 82 Phl p 5 reactive TCCs were isolated from the peripheral blood of 18 patients allergic to timothy grass pollen. The number of TCC per individual varied from one to 11. Phenotype analysis of all TCCs revealed the TCR α/β type and the T-helper phenotype ($\text{CD}3^+$, $\text{CD}4^+$, $\text{CD}8^-$). The specificity of TCCs for nPhl p 5 and additionally the stimulatory properties of two recombinant isoforms of Phl p 5 were first assessed. Based on a stimulation index > 10 , all 82 TCCs proliferated in response to stimulation with purified nPhl p 5, 22 of 73 (30%) reacted with rPhl p 5a and 42 of 80 (53%) reacted with rPhl p 5b (see Table 2). Thirteen TCCs recognized both of the recombinant isoforms. Twenty-six TCCs failed to recognize any of the recombinant allergens despite their reactivity with nPhl p 5.

Table 3. Distribution of T cell reactive regions on the Phl p 5b allergen identified with 52 TCCs from 13 donors

Region	AAs on Phl p 5b	Number of TCCs/donors
A	181–207	28 TCCs (54%)/8
B	133–150	9 TCCs (17%)/6
C	16–48	5 TCCs (10%)/5
Elsewhere	6 epitopes	10 TCCs (19%)/7

Table 4. Proliferative responses of five selected TCCs reactive with Phl p 5 epitopes 136-147/161-172 (a) and 184-195/209-220 (b) to peptides synthesized according to corresponding regions of Phl p 5b and Phl p 5a

	JR 6	JR 7	DW 8	UZ 24	UZH 2
(a)					
Control (without peptide)	50	75	15	25	30
L Q I I D K I D A A F K (Phl p 5b)	1580	2900	1030	50	1260
L Q V I E K V D A A F K (Phl p 5a)	580	1610	350	2190	20
	DW 28	JR 15	CB 10	AS 5	KS 3
(b)					
Control (without peptide)	65	90	60	15	100
Y K C I P S L E A A V K (Phl p 5b)	1250	3400	1960	780	1700
Y K F I P A L E A A V K (Phl p 5a)	3210	3300	40	1630	1100

c.p.m., counts per minute after ^3H -TdR uptake.

By using 178 overlapping dodecapeptides covering the rPhl p 5a as well as rPhl p 5b sequences (see Fig. 1), 19 T-cell epitopes could be identified (see Table 2). Many TCCs were stimulated by two neighbouring overlapping dodecapeptides. Interestingly, four of the peptides induced proliferative responses in 32 TCCs from several patients (see Table 2): seven individuals harboured T-cell specificities for amino acids 209–220 (Phl p 5a)/amino acids (AAs) 181–195 (Phl p 5b), and six individuals for AAs 161–172 (Phl p 5a)/AAs 136–150 (Phl p 5b), respectively. A total of 42 TCCs from 52 mapped TCCs (81%) recognized three T-cell reactive regions on the Phl p 5b allergen (see Table 3). In each case two donors recognized the epitope 232–243 on the Phl p 5a molecule as well as epitopes 49–63, 97–111, 163–174, 190–204 and 196–207 on the Phl p 5b molecule. The remaining epitopes were found once each in different subjects. Individual patients recognized 1–6 specificities on the allergen. Although seven TCCs (JR 10, KSE 19, KSE 32, GE 3, GE 9, USR 11 and USR 12) strongly reacted with rPhl p 5a or rPhl p 5b, no significant stimulation was measured with any of the dodecapeptides.

Proliferative responses of 10 representative TCCs reacting with two major epitopes (A and B) are shown in Table 4. Five TCCs from four different donors were tested with two peptides (displaying three AA exchanges) derived from homologous sequences (isoepitopes) of Phl p 5a and Phl p 5b. Among them three TCCs (JR 6, JR 7 and DW 28) recognized both peptides, though lower stimulation indices with the Phl p 5a derived peptide were observed. The other two TCCs were specific for the Phl p 5a (UZ 24) as well as Phl p 5b (UZH 2) peptides. Five additional TCCs from five different donors reacting with two peptides (displaying two AA exchanges) representing a further isoepitope of Phl p 5a

and Phl p 5b were tested. With the exception of TCC CB 10, which was specific to the Phl p 5b peptide, four TCCs demonstrated a simultaneous recognition of both peptides. All 10 selected TCCs (Table 4) presented their peptide by HLA-DR molecules as shown by the fact that monoclonal antibodies directed against framework antigens of the HLA-DR region molecules blocked nPhl p 5-dependent proliferation (data not shown). A dominant HLA-DR haplotype could not be identified among the 15 donors tested (see Table 1), although in three instances two donors without family connection showed the same HLA-DR haplotypes (KS and UZ; AH and GE; and AS and USR).

IL-4 and γ -IFN secretion from 50 TCCs with identifiable epitope specificity were analysed after specific stimulation with nPhl p 5 (see Table 5). Eight TCCs revealed a high IL-4: γ -IFN ratio (>10), typical of TH₂ cells, whereas 25 TCCs could be classified as TH₀ clones (secretion of both lymphokines). Twelve TCCs demonstrated a low IL-4: γ -IFN ratio (<0.1), typical of TH₁ cells. The remaining five TCCs produced neither IL-4 nor γ -IFN. Furthermore 38 of the 50 TCCs were tested for IL-13 production. All TCCs secreted IL-13, among them 34 TCCs which produced concentrations of at least 500 pg/mL.

Discussion

A number of recent studies have investigated T-cell epitopes of the group 1 grass allergens [21–24]. Information about T-cell activating epitopes of group 5 allergens is still incomplete. Zhang *et al.* [15] described potential T-cell epitopes on group 9 (5) allergens of Kentucky blue grass pollen identified by proliferation studies using freshly isolated PBMC of grass allergic donors. Blaher *et al.* [14]

Table 5. Cytokine production of 50 Phl p 5-reactive TCCs.

Epitope AAs on: rPhl p 5a	rPhl p 5b	TCC	IL-4 (pg/mL)	γ -IFN (pg/mL)	IL-13 (pg/mL)	T-helper subset ^a
53–64	25–36	CB7	<3	<1	n.t.	?
	31–42	JS1	350	50	n.t.	0
	37–48	GE12	80	690	>2500	0
	43–54	KSE18	20	100	1200	0
	46–57	KSE 9	70	180	900	0
		KSE 29	20	50	1500	0
	49–60	JR 11	40	80	n.t.	0
	49–63	MD 6	90	10	n.t.	0
		USR 6	20	700	1340	1
	97–111	JR 24	90	< 1	1200	2
	100–111	UZ 25	70	40	1200	0
	112–123	UD 6	25	1500	>2500	1
	136–150	GE 4	10	< 1	160	2
		GE 7	<3	50	260	1
		UZH 2	90	30	600	0
161–172	136–150	DW 8	170	< 1	2200	2
161–172		JR 6	1900	> 2500	>2500	0
		JR 7	280	1080	>2500	0
		JR 19	> 2000	> 2500	1200	0
		UZ 24	10	22	700	0
	163–174	AS 3	15	350	1150	1
209–220		MD 5	10	8	n.t.	0
	181–195	CB 2	600	200	>2500	0
		CB10	< 3	< 1	>2500	?
		CB14	90	< 1	n.t.	2
	184–195	MD 1	310	45	n.t.	0
		MD 2	80	15	n.t.	0
		MD 3	210	20	n.t.	2
	181–195	DW 16	< 3	< 1	n.t.	?
		DW 23	140	< 1	80	2
		DW 25	40	< 1	900	2
		DW 28	850	360	300	0
		JR 9	110	400	>2500	0
		JR 13	1600	> 2500	>2500	0
		JR 15	1900	> 2500	>2500	0
		JR 25	140	4900	>2500	1
232–243		JR 27	< 3	750	>2500	1
		KS 1	< 3	2100	>2500	1
		KS 2	< 3	650	1000	1
		KS 3	< 3	> 2500	>2500	1
		KS 4	< 3	800	1500	1
		KS 5	< 3	> 2500	>2500	1
		AS 4	< 3	<1	500	?
		AS 5	< 3	80		
	190–204	CB 1	330	< 1	n.t.	2
	196–207	DW 14	< 3	< 1	n.t.	?
	211–225	CBH 1	60	30	1200	0
		CBH 10	20	60	2200	0
		UZ 6	15	70	640	0
		UZH 6	190	200	>2500	0

a: Classification of T-helper subsets: TH₁ cells, low IL-4: γ -IFN ratio (< 0.1) TH₂ cells, high IL-4: γ -IFN ratio (> 10.0). n.t., not tested.

described only six TCCs isolated from one atopic person responding to defined peptides spanning the entire sequence of a Lol p 9/5 isoform. We used 178 dodecapeptides, synthesized according to the sequence of two recombinant expressed isoforms of Phl p 5a/b to map T-cell epitopes of 82 TCCs established from 18 allergic individuals. These TCCs were derived from individual TCLs obtained following stimulation with natural Phl p 5 allergens containing a mixture of various isoforms. In line with reports on group 1 allergens, multiple T-cell epitopes associated with group 5 allergens were detected. However, 24 of the TCCs did not recognize any of the synthetic peptides employed. It is safe to say that contaminants (other allergens) of the natural group 5 allergens are not the back-ground for this phenomenon. Above all, the purified Phl p 5 allergens did not contain group 1 (Phl p 1) glycoproteins as tested by a monoclonal antibody immunoassay (sensitivity of 10 ng/mL) for quantitative analysis of group 1 allergens established in the authors' laboratory. Furthermore, carbohydrates cannot play a role as group 5 allergens are not glycosylated. Spiegelberg *et al.* suggested that many TCCs which proliferated strongly to natural Lol p 1 may not react with any of the synthetic peptides used to map T-cell epitopes on the Lol p 1 allergens, because the TCCs recognize epitopes or isoepitopes from other Lol p 1 isoforms [24]. In a study in which all TCLs were selected by means of a single recombinant Phl p 1 isoform, and not a mixture of isoforms as in a natural allergen preparation, specific T-cell epitopes for 39 of 40 Phl p 1 reactive TCCs could be identified [21]. Recently, Ferreira *et al.* [25] demonstrated that some patients display a broad range of T-cell recognition patterns for Bet v 1, the major allergen of birch pollen, whereas T cells from other subjects seem to be restricted to particular isoforms. Furthermore, Løwenstein and co-workers also reported the significance of isoallergenic variations [26]. The data presented here support their observations, and the fact that 18 of the 24 TCCs that failed to react with peptides reacted only with nPhl p 5 and not the recombinant proteins strongly suggest that some of donors in this study favour particular isoforms of Phl p 5 whereas others demonstrate a broader recognition pattern.

Allergens are designated as isoallergens when they have at least 67% of the amino acid sequence in common [27]. Sequence alignments reveal that group 1 grass pollen allergens show a strikingly high identity of about 90% and a homology of about 94% [28]. In the case of group 5 allergens, the structural properties are more diverse. These allergens, even sorted into group 5a and 5b, display alignment identities of only 70–80% and homologies in the range of 80–90% [10]. Phl p 5a and Phl p 5b may represent different group entities as the allergens differ in molecular weight by 6 kDa and in their N-terminal structures by 40%. Interestingly, in sweet velvet grass (*Holcus lanatus*) only the

Phl p 5b analogue is expressed [29]. Considering these specific conditions, the differentiated recognition pattern of the TCCs in this study is explicable.

However, interpretation of the results of some of the proliferation tests is more hypothetical: six TCCs were not stimulated by any of the synthetic peptides though they recognized recombinant Phl p 5 allergens. Conceivably, the synthetic peptides were processed differently from the native allergen, so that they no longer form structures recognized by the T cells. Furthermore, it might be that some of the TCCs require longer peptides. Carballido *et al.* reported that an additional screening of selected TCCs by using octadecapeptides rather than dodecapeptides permitted the identification of additional T-cell epitopes on phospholipase A₂, the major allergen of bee venom [30]. It should also be considered that the efficiency of scanning of T-helper cell epitopes using pools of synthetic peptides can be influenced by various factors, especially the length and endings (blocked or unblocked) of peptides, the number of available antigen presenting cells and the peptide concentration [31]. In one of the donors (KS) the TCC reacted with only two corresponding peptides, suggesting that this epitope could be dominant in this patient. Recently various other authors described similar individual-restricted T-cell repertoires for other allergens [21,32].

In spite of group 5 allergen heterogeneity, three distinct epitope regions were recognized by several patients. A total of 42 TCCs (81%) reacted with these T-cell stimulating regions on the Phl p 5b molecule. Phl p 5a-crossreactive and non-crossreactive TCCs were observed. For some TCCs, certain exchanges of AAs within the homologous peptides were unimportant, whereas for other TCCs these differences correlated with a decreased reactivity or a lack of proliferation response. Similar variations in reactivities of allergen-specific TCCs with four recombinant isoforms of Cor a 1 (the major allergen of hazel pollen) were found by Schenk *et al.* [33].

Of the 50 TCCs investigated, 50% could be classified as TH₀ clones, 24% as TH₁ clones and 16% as TH₂ clones. Five clones secreted neither IL-4 nor γ -IFN. No correlation was found between peptide epitope reactivity and lymphokine secretion pattern of the TCC. Also, other authors described a dominant TH₀-like subset for allergen reactive TCCs [34,35]. For instance, 19 of 33 TCCs (57%) isolated from allergic individuals by Ebner and co-workers displayed a TH₀-like pattern after stimulation with Bet v 1, the major birch pollen allergen [35].

Significant concentrations of IL-13 were produced by all of 38 TCCs tested, independent of IL-4 or γ -IFN. Essayan and co-workers recently showed that there was no quantitative correlation between the levels of IL-4 and IL-13 in short ragweed allergen reactive TCCs [36]. Therefore, a classification of the cytokine phenotypes of TCCs based

exclusively on the ratio of IL-4 to γ -IFN is problematic and too simple. IL-13 and IL-4 interact with a common receptor component required for signal transduction and show similar biological activities on human and mouse monocytes/macrophages and human B cells [37]. In addition, IL-13 induces expression of germline ϵ transcripts and switching to IgE in immature B cells and naive surface IgD⁺ B cells, as well as the expression of the CD23 molecule on B cells and monocytes [37]. Recently, it has been demonstrated that IL-13 can be produced by TCCs belonging to CD4⁺ or CD8⁺, TH(C)₀⁻, TH(C)₁⁻ and TH(C)₂-like subsets, following activation by specific antigen or polyclonal stimulation [38]. The same authors found that IL-13 production was induced rapidly and was relatively long lasting in peripheral blood T cells and TCCs, whereas IL-4 production by the same cells was transient. Since the immunological activities of IL-13 on B cells and monocytes are similar to those of IL-4, one may suppose that despite a low IL-4 production, IL-13-secreting TCCs play a role for the maintenance of the group 5 specific IgE response *in vivo*.

Taken together the data presented here suggest that Phl p 5 allergens contain a number of T-cell epitopes that vary between individuals. However, certain epitope regions were recognized more frequently by T cells of different patients. T-cell-stimulating epitopes and non-stimulating regions can be found at corresponding regions of different isoforms of the Phl p 5 allergen. From a therapeutic point of view, selected immunodominant T-cell epitopes (peptides) may be considered as potential immunotherapeutic tools, e.g. peptides of cat dander or house dust mite allergens [39,40]. Obviously an effective immunotherapy may require the use of a minimum number of immunodominant peptides to establish a regulatory network (e.g. by cross epitope suppression), that control the response to the allergen as whole [39]. As grass pollen contains at least two major allergens (groups 5 and 1) the situation is more complex. Therefore, on the basis of these and other findings [21] there is a need for investigations in animal models and therapeutic studies. In this way potential peptides could be tested for cross-regulatory effects of tolerance induction which could target the allergen-specific T-cell compartment.

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