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Analysis of Human T Cell Clones Reactive with Group V Grass Pollen Allergens

Key Words

Cross-reactivities
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T cell clones

Abstract

Twenty-seven T cell clones (TCC) reactive with group V allergens of *Phleum pratense* (*Phlp V*) were established from the peripheral blood of 3 patients allergic to grass pollen. Twenty-four TCC showed the helper cell phenotype CD3+, CD4+, CD8–; the remaining clones were CD3+CD4–CD8+. T cell recognition of *Phlp V* was exclusively HLA-DR restricted. Many of the *Phlp V* reactive TCC (19 of 27; 70%) were stimulated additionally by other group V allergens isolated from *Lolium perenne*, *Poa pratensis*, and *Dactylis glomerata*. These data indicate the existence of cross-reacting T cell epitopes among group V allergens of different grasses. The *Phlp V* triggered cytokine production demonstrated in 13 out of 24 CD4+ TCC a Th₂-like pattern (high interleukin 4/γ-interferon ratios) suggesting group V allergens as important targets of grass pollen specific IgE.

Introduction

Grass pollen allergens trigger typical symptoms of the widespread pollinosis such as conjunctivitis, rhinitis, and asthma bronchiale. The responsible allergenic components can be classified into different groups according to their physicochemical and immunological properties [1]. Group I and V allergens are of special importance due to their high sensitization index [1–3]. Allergen-specific immune responses can be merely understood, if more detailed information is available about T cell epitopes regulating specific IgE production [4].

Therefore, many appropriate investigations on the allergen-specific T cell level have been performed in the meantime. In case of grass pollen allergens most data were published about group I–III allergens of *Lolium perenne* [5, 6] and group V/IX allergens of *Poa pratensis* [7, 8].

Some time ago we studied group V allergens on the antibody level, using suitable murine monoclonal antibodies [9–11]. Now we established T cell clones (TCC) from 3 dif-

ferent allergic donors reactive to group V allergens from timothy grass *Phleum pratense*; (*Phlp V*). These TCC were characterized regarding their cross-reactivities to other relevant group V allergens as well as their lymphokine secretion pattern after *Phlp V* stimulation.

Materials and Methods

Allergens

The following lyophilized grass pollen extracts were generously provided by Allergopharma (Reinbek, Germany): *P. pratense* (timothy), *L. perenne* (ryegrass), *P. pratensis* (Kentucky bluegrass), and *Dactylis glomerata* (orchard grass).

Group V allergens were isolated as described in detail elsewhere [11, 12] by immunoaffinity chromatography using monoclonal antibody 1D11 binding to a common epitope of group V allergens of various grasses [9–11]. After purification, all group V allergens were homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The degree of purity was estimated to be 94–96% by scanning using laser densitometer.

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Patients

Atopic status with grass pollen allergy was defined by typical anamnesis, positive skin prick tests, and serum-specific IgE as measured by radioallergosorbent test (class III or IV). None of the 3 patients (FR, male, age 42 years, HLA: DR 8/13, DRw 52, DQw 1/4; DW male, age 35 years, HLA: DR 15, DQw 1; CB female, age 21 years, HLA: DR 4/17, DRw 52/53, DQw 2/8) were receiving corticosteroid therapy. All 3 patients had undergone allergen-specific immunotherapy (Allergovit®; Allergopharma, Germany; an alum-adsorbed mixture of six grass extracts): patients FR and DW 1 year and patient CB 2 years before establishing TCC. In all 3 patients immunotherapy was successful on the basis of symptom score as well as the consumption of antiallergic drugs. In each case peripheral blood mononuclear cells (PBMC) were obtained during the grass pollen season.

Isolation of *PhlpV* reactive TCC

Briefly, PBMC (2×10^6 /well) were stimulated in 24-well (final volume 1 ml) flat-bottom plates (NUNC, Roskilde, Denmark) using an optimal concentration of purified *PhlpV* (5–10 µg/ml) for 7 days in RPMI-1640 medium (Gibco, Paisley, UK) supplemented with 2 mmol/l *L*-glutamine (Sigma, St. Louis, Mo., USA), 1% v/v antibiotic-antimycotic solution (Sigma), and 10% inactivated human AB serum (Sigma). After a further stimulation period of 7 days in the presence of interleukin 2 (IL-2; 25 IU/ml; Pharma Biotechnology, Hannover, Germany), lymphoblasts were enriched on density gradient (Lymphoprep; Nycomed, Oslo, Norway). Then a second stimulation cycle of 10 days with 5×10^5 lymphoblasts/well in the presence of irradiated (3,000 rad) autologous PBMC (5×10^5 /well), *PhlpV* (10 µg/well), and IL-2 (25 IU/well) followed.

The *PhlpV* reactive T cell lines obtained were tested regarding the *PhlpV* specificity by a tritiated methylthymidine (^3H -TdR; Amersham, UK) incorporation assay as described under Proliferation Assays. Thereafter, suitable T cell lines were cloned (0.3 cells/well, final volume 0.2 ml) in 96-well round-bottomed plates (NUNC) in the presence of irradiated (3,000 rad) allogeneic PBMC (5×10^5 /well), phytohemagglutinin (1 µg/ml; Biochrom, Berlin, Germany), and IL-2 (25 IU/ml).

After 14 days microcultures were fed with fresh irradiated allogeneic PBMC, phytohemagglutinin, and IL-2. Furthermore, fresh IL-2 (25 IU/ml) was added at 4- to 5-day intervals. Before performing allergen-specific proliferation assays, the clones were settled for almost 10 days after the last addition of irradiated allogeneic PBMC. Selected *PhlpV* reactive TCC (5×10^3 /well) were further expanded in 24-well plates (NUNC; final volume 1 ml) by restimulation with phytohemagglutinin (2.5 µg/ml), irradiated allogeneic feeder cells (5×10^5 /well), and IL-2 (25 IU/ml).

Proliferation Assays

The specificities of TCC were assessed as follows: T cell blasts (in each case 2×10^4 cells/well) were stimulated in triplicate cultures in 96-well round-bottomed plates (NUNC, final volume 0.2 ml) with different group V allergens (1, 10, and 20 µg/ml) in the presence of irradiated autologous PBMC (5×10^4 /well) as antigen-presenting cells. After 56 h ^3H -TdR (1 µCi/well) was added for 16 h. Proliferation (^3H -TdR incorporation) was measured using a Matrix 96 beta counter (Canberra Packard, Meriden, Conn., USA); the results are expressed as arithmetic mean counts per minute (cpm) for triplicate cultures. Responses were considered positive when the stimulation index (ratio between cpm obtained in cultures with TCC plus irradiated autologous PBMC plus group V allergens and the same cultures without group V allergens) was greater than 10.

The standard error of the mean value for all experiments was <20%. As negative controls, T cell blasts alone, irradiated allogeneic PBMC, and an irrelevant antigen (tetanus toxoid, 10 µg/ml; Behringwerke, Marburg/Lahn, Germany) were tested simultaneously. In each case the data from representative experiments are shown.

Proliferation Inhibition Experiments

For testing of the HLA class II restriction of TCC, monoclonal antibodies (against HLA-DR, HLA-DP, or HLA-DQ; Becton Dickinson, San Jose, Calif., USA) were added (5 µl/ml) to *PhlpV* (10 µg/ml) driven T cell proliferation. The ^3H -TdR incorporation of TCC in the absence and presence of different HLA class II specific monoclonal antibodies was compared and expressed as percent inhibition (for calculation see legend to table 4).

Phenotype Analysis of TCC

Analysis of TCC for expression of cell surface markers CD3, CD4, and CD8 was performed by flow cytometry using appropriate fluorescein isothiocyanate labeled monoclonal antibodies (Becton Dickinson) and FACScan (Becton Dickinson).

Cytokine Production of TCC

PhlpV stimulated cytokine production (IL-4 and gamma interferon – γ -IFN – for all TCC, IL-2 for selected TCC) was measured using commercial enzyme-linked immunosorbent assay kits (IL-4: R/D Systems, Minneapolis, Minn., USA; γ -IFN: Laboserv, Gießen, Germany; IL-2: R/D Systems) according to the manufacturers' instructions. In each case 1×10^6 TCC per well (free of residual IL-2) were added to 1×10^6 irradiated autologous PBMC per well in the absence or presence of 10 µg/ml *PhlpV* in 24-well flat-bottomed plates (NUNC, final volume 1 ml). After incubation for 36 h, the cells were centrifuged, and culture supernatants were collected, filtered (0.22 µm), and stored in aliquots at –20°C until testing. As controls supernatants from cultures containing irradiated feeder cells alone were used. The cytokine concentrations were regarded as positive if the values were at least 5 standard deviations above those of control supernatants.

Results

Altogether 27 *PhlpV* reactive TCC (5 from donor FR, 12 from donor DW, 10 from donor CB) were selected after cloning of three T cell lines and analyzed in detail.

Flow Cytometric Analysis

FACS analysis revealed the T cell phenotypes CD3+CD4+CD8– for 24 TCC and CD3+CD4–CD8+ for 3 TCC (all from donor CB: CB11, CB12, CB15).

Group V Specific Proliferation

During primary screening 27 TCC were selected with regard to their proliferative response to purified *PhlpV* (stimulation index >10). Thereafter all TCC were tested with other purified group V allergens *LolpV*, *PoapV*, and *DacgV* using specific proliferation assays. The following control assays were performed, with inconspicuous results:

Table 1. Proliferative responses of group V reactive TCC: 'mono-reactive clones' (*Phl p V* specific)

TCC	³ H-TdR, cpm×10 ³				
	negative control ^a	<i>Phl p V</i>	<i>Lol p V</i>	<i>Poa p V</i>	<i>Dac g V</i>
FR6	0.10	2.20	0.32	0.36	0.44
FR12	0.50	4.60	1.30	2.40	1.80
DW11	0.18	1.90	0.20	0.28	0.12
DW14	0.03	2.70	0.04	0.10	0.06
DW16	0.04	1.80	0.22	0.07	0.08
DW26	0.45	4.20	1.90	1.20	1.70
CB2	0.03	1.00	0.06	0.20	0.04
CB14	0.02	0.28	0.12	0.12	0.05

For each clone the maximum ³H-TdR incorporation after stimulation with different concentrations (1, 10, or 20 µg/ml) of purified group V allergens is listed. Positive responses in italics (stimulation index > 10). The standard error of the mean for all experiments was <20%. Data were obtained from several comparable experiments.

^a Irradiated autologous antigen-presenting cells plus TCC in the absence of group V allergens.

Table 2. Proliferative responses of group V reactive TCC: 'oligo-reactive clones' (reactivities with two group V allergens)

TCC	³ H-TdR, cpm×10 ³				
	negative control ^a	<i>Phl p V</i>	<i>Lol p V</i>	<i>Poa p V</i>	<i>Dac g V</i>
FR8	0.15	1.60	0.70	2.20	0.30
DW1	0.40	8.20	0.07	4.40	0.11
DW8	0.22	6.60	1.30	0.52	2.80
DW23	0.11	5.80	0.13	1.10	0.26
DW25	0.16	4.20	0.25	1.75	0.65
CB1 ^b	0.04	0.56	0.25	0.60	0.24

For further explanation see table 1.

^a See table 1.

^b CD8+ TCC.

Table 3. Proliferative responses of group V reactive TCC: 'multi-reactive clones' (reactivities with at least three group V allergens)

TCC	³ H-TdR, cpm×10 ³				
	negative control ^a	<i>Phl p V</i>	<i>Lol p V</i>	<i>Poa p V</i>	<i>Dac g V</i>
FR2	0.20	7.70	9.20	9.40	14.40
FR7	0.25	3.00	2.70	2.50	0.70
DW24	0.10	7.30	2.20	2.00	4.40
DW28	0.15	6.00	4.00	1.70	2.70
DW29	0.13	4.80	0.93	2.00	1.38
CB1	0.10	3.10	0.50	0.50	4.20
CB4	0.12	4.80	0.60	1.20	1.70
CB10	0.04	2.70	0.50	3.10	0.10
CB13	0.05	0.60	0.15	3.00	1.60
CB3	0.06	3.20	0.15	0.90	0.65
CB7	0.02	0.30	0.20	0.20	0.40
CB12 ^b	0.02	0.40	0.10	0.30	0.80
CB15 ^b	0.07	0.70	1.40	1.40	1.40

For further explanation see table 1.

^a See table 1.

^b CD8+ TCC.

(1) TCC plus irradiated autologous antigen-presenting cells without allergen (see tables 1–3); (2) TCC plus irradiated autologous antigen-presenting cells plus an irrelevant antigen (tetanus toxoid, 10 µg/ml; data not shown), and (3) TCC plus irradiated allogeneic antigen-presenting cells plus *Phl p V* (10 µg/ml; data not shown).

Various groups of TCC appear after analysis of all proliferation assays due to their proliferation patterns. Whereas some TCC are only 'monoreactive' (*Phl p V* specific, table 1) the majority of TCC was cross-stimulated (tables 2, 3) by other group V allergens. Reactivities to 2–4 group V grass pollen allergens have been found.

All 24 CD4+ TCC were HLA-DR-restricted. Concerning HLA restriction, selected data are shown in table 4.

Cytokine Production

The cytokine production of all TCC after stimulation with *Phl p V* was measured (tables 5–7). Cytokine concentrations were regarded as positive if the values were 5 standard deviations above those of control supernatants (derived from irradiated autologous antigen-presenting cells alone). In control supernatants of all 3 donors, the concentrations of IL-4, γ-IFN, and IL-2 were lower than the minimum detectable amounts (3 pg/ml for IL-4 and 20 pg/ml for γ-IFN and IL-2) of the assays used. Furthermore, a spontaneous cytokine release (irradiated antigen-presenting cells plus TCC without allergen) was not detectable. Allergen (*Phl p V*) specific stimulation of 24 CD4+ TCC revealed a high IL-4/γ-

IFN ratio: IL-4 > 100 pg/ml, γ-IFN < 20 pg/ml for 13 (54%) clones (Th₂-like pattern). IL-4 as well as γ-IFN were found in 2 of 24 supernatants (Th₀-like pattern), whereas in 6 of 24 supernatants both lymphokines were not detectable. This 6 TCC (table 6) were tested additionally for allergen-triggered IL-2 production. Two of the 6 TCC demonstrated a significant IL-2 secretion. Two of 3 CD8+ TCC (table 7) produced IL-4 and γ-IFN (Th₀-like pattern); the remaining TCC showed no lymphokine production.

Table 4. Inhibition of *Phl p V* specific proliferation by anti-HLA monoclonal antibodies: presentation of ten selected TCC

TCC	³ H-TdR, cpm×10 ³				
	no monoclonal antibody	anti-HLA-DP	anti-HLA-DQ	anti-HLA-DR	% inhibition of anti-HLA-DR ^a
DW25	2.11	2.00	1.70	0.03	98.6
DW29	1.40	1.00	1.20	0.02	98.6
DW28	1.60	1.60	1.20	0.01	99.4
DW23	2.10	1.30	1.60	0.03	98.6
CB4	0.40	0.40	0.30	0.07	82.5
CB15	0.50	0.30	0.25	0.07	86.0
CB2	1.00	0.70	0.70	0.16	84.0
CB14	0.18	0.12	0.16	0.03	83.3
FR2	7.70	7.40	7.60	0.40	94.8
FR7	3.30	2.80	2.70	0.35	88.3

Phl p V reactive TCC (2×10⁴/well) were stimulated with *Phl p V* (10 µg/ml) and autologous PBMC (5×10⁴/well) in the presence or absence of anti-HLA class II blocking monoclonal antibodies.

^a % inhibition by anti-HLA-DR antibody is expressed as:

$$\% \text{ inhibition} = \frac{\text{cpm} (- \text{antibody}) - \text{cpm} (+ \text{antibody})}{\text{cpm} (- \text{antibody})} \times 100\%$$

Table 5. Cytokine production of CD4+ TCC stimulated with *Phl p V*

No.	TCC	IL-4 pg/ml	γ-IFN pg/ml	No.	TCC	IL-4 pg/ml	γ-IFN pg/ml
1	DW28	1,026	<20	13	DW1	150	<20
2	FR2	960	<20	14	DW25	82	<20
3	CB3	926	<20	15	DW29	32	<20
4	FR8	773	<20	16	CB10	23	<20
5	DW11	589	<20	17	FR6	1,920	4,520
6	CB2	473	<20	18	DW26	>2,000	3,557
7	FR12	448	<20	19	DW14	<3	<20
8	CB14	367	<20	20	DW16	<3	<20
9	FR7	350	<20	21	DW24	<3	<20
10	CB1	326	<20	22	CB7	<3	<20
11	DW23	209	<20	23	CB13	<3	<20
12	DW8	171	<20	24	CB4	<3	<20

Supernatants were obtained by stimulating TCC (1×10⁶/well, final volume 1 ml) in the presence of 10 µg/ml *Phl p V* and irradiated autologous PBMC (1×10⁶/well) for 36 h. The cytokine concentrations were regarded as positive if the values were at least 5 standard deviations above those of control supernatants obtained from irradiated autologous antigen-presenting cells alone.

Discussion

Besides group I, group V allergens are the most important major allergens of grass pollen [1–3]. In the meantime information about B cell epitopes of group V allergens including cross-reactivities on the IgE level has been improved [2, 3, 9–11]. On the contrary, data on T cell epitopes

of group V allergens are rare [7, 8]. Therefore, we characterized a panel of 27 *Phl p V* reactive TCC established from the peripheral blood of 3 typical pollinosis patients. As expected, the majority of TCC (24/27) showed the helper cell phenotype CD3+CD4+CD8–, whereas the remaining three clones were CD3+CD4–CD8+. Others [e.g., 13, 14] reported on isolation of CD3+CD4–CD8+ allergen reactive TCC

Table 6. IL-2 production of six selected CD4+ TCC stimulated with *Phlp V*

No.	TCC	IL-2 pg/ml
19	DW14	<20
20	DW16	350
21	DW24	<20
22	CB7	<20
23	CB13	<20
24	CB4	475

For further explanation see table 5.

Table 7. Cytokine production of CD8+ TCC stimulated with *Phlp V*

TCC	IL-4 pg/ml	γ -IFN pg/ml
CB11	449	410
CB12	830	1,910
CB15	<3	<20

For further explanation see table 5.

too. All three CD8+ TCC came from the same donor (CB). Perhaps CD8+ allergen specific TCC arose from the more prolonged (2 years) immunotherapy as compared with the other 2 donors.

The T cell recognition of *Phlp V* was always HLA-DR restricted. Obviously HLA-DR molecules restrict T cell responses of a majority of nominal allergens [e.g., 14–16]. Specific proliferation assays with various purified group V grass pollen allergens allow several conclusions: The 'T cell repertoire' directed at *Phlp V* allergens encloses species (*P.pratense*) specific as well as cross-reacting epitopes (see tables 1–3). Interestingly, 19 TCC seem to recognize common/cross-reacting epitopes or structural motifs [17] of amino acid sequences on different group V allergens. In the meantime first data about such homologies between *Phlp V* and *Poa p V/IX* (90% identities) as well as between *Phlp V* and *Lolp V/Ib* (35% identities) have been published [18]. Obviously, the higher degree of sequence homologies of *Phlp V* to *Poa p V* in comparison with *Lolp V* will be confirmed analyzing the proliferative responses: 17 of 19 cross-reacting TCC were reactive to *Poa p V* too, whereas only 7 of these clones recognized *Lolp V* additionally (see tables 2 and 3). Similar structural homologies seem to exist between

Phlp V and *Dac g V*, as 12 of 19 cross-reacting TCC proliferate in the presence of *Dac g V* to.

Some of the *Phlp V* reactive TCC (FR2, FR8, CB1, and CB10) recognized common epitopes of other group V allergens even more effective than for *Phlp V* itself. Perhaps minor variations of amino acid sequences/structural motifs on homologous epitopes could be the reason for this phenomenon. Recently, comparable data have been published for immunodominant epitopes on corresponding major allergens of different tree pollens [19]. Previously the existence of intraspecies (*Lolp I, II, and III*) and even interspecies (*Poa p V, Lolp I*) T cell epitopes on grass pollen has been described [6, 8]. Now appropriate investigations regarding some cross-recognitions of our group V reactive TCC of intraspecies (*Phlp I*) as well as interspecies (*Lolp I*) group I allergens are under way [manuscript in preparation].

Besides we were especially interested in the functional properties of the CD4+ TCC. Therefore, the production of IL-4 and γ -IFN after allergen-specific (*Phlp V*) stimulation was tested. We preferred this stimulation procedure, as pathways which circumvent triggering the T cell receptor/CD3 complex are not representative of the natural situation [e.g., ref. 15]. Thirteen of 24 CD4+ TCC demonstrated higher IL-4/ γ -IFN ratios (Th₂-like pattern; see table 5, Nos 1–13). Interestingly, among these clones nine belong to cross-reacting TCC (see tables 1, 2 and 3). Such cross-recognition triggering IL-4 secretion could be responsible for an 'amplification' of IgE responses to homologous major allergens derived from various grass species. Two other CD4+ TCC showed a Th₀-like cytokine pattern (FR6 and DW 26), whereas six clones (Nos. 19–24; table 5) produced neither IL-4 nor γ -IFN. As 2 of these 6 clones secreted IL-2 (table 6), they belong to Th₁-like T helper cells. However, 4 TCC seem to be indeed 'nonproductive'. Perhaps such clones may represent so-called precursor T helper cells ('Th_p') isolated from atopic donors being in remission, as shown recently by Spiegelberg et al. [20]. The results of in vitro cytokine assays could be estimated as follows: The frequent Th₂-T helper cell status confirms indeed the real in vivo situation of all 3 allergic patients at the time of TCC isolation, in the midst of grass pollen season. Similar data were published for many other allergen-specific TCC also by others [4]. On the other hand, a random selection of TCC has happened which is less representative of the clinical situation, as all 3 donors have already undergone specific immunotherapy. Therefore, future investigations should include TCC established from the same allergic donors before and after (remission) immunotherapy. Furthermore, analysis of grass pollen specific T cell responses of nonallergic persons [e.g., ref. 20] would be desirable. But all our at-

tempts to isolate group V reactive T cell lines from healthy persons failed throughout. Nevertheless, the presented data obtained from this panel of group V reactive TCC allow the following conclusions: The exact identification of cross-reacting (common) T cell epitopes on group V major grass pollen allergens could be very sensible due to the critical

role of T cells in the regulation of allergen-specific IgE responses [21]. This knowledge could be utilized to test potential (immunodominant?) peptides concerning immune mechanisms leading to an allergen-specific immunotolerance for a scientifically based immunotherapy [21, 22].

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