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## Group 5 Allergens of Timothy Grass (Phl p 5) Bear Cross-Reacting T Cell Epitopes with Group 1 Allergens of Rye Grass (Lol p 1)

### Key Words

T cell clones  
Cross-reactivities  
Proliferation assays  
Grass pollen allergens, group 5  
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### Abstract

Selected human T cell clones reactive with group 5 allergens of timothy grass (Phl p 5) were cross-stimulated in specific proliferation assays with group 1 allergens of rye grass (Lol p 1). Such interspecies cross-reactivities result obviously from structural motifs presented on defined Phl p 5 fragments as shown with recombinant Phl p 5 products.

### Introduction

Group 5 and 1 grass pollen allergens are of special importance in the allergy field due to their outstanding sensitization index [1, 2]. So far only a few studies investigated the group 5 and 1 specific T cell level [3–5]. Recently we reported on human T cell clones (TCC) reactive to various group 5 grass pollen allergens [6].

We now present evidence that a certain percentage of Phl p 5-reactive TCC also proliferated in the presence of Lol p 1. By means of recombinant Phl p 5 products [7] this phenomenon was characterized in more detail.

### Material and Methods

#### Natural Allergens

Phl p 5 allergens were isolated as described in detail elsewhere [8] by immunoaffinity chromatography using the monoclonal antibody 1D11 binding to a common epitope of group 5 allergens of various grasses [9]. Lol p 1 was purified [10] by reversed-phase high-pressure liquid chromatography (HPLC; Shimadzu, Tokyo, Japan). Briefly, *Lolium perenne* extract was diluted in 0.1% trifluoroacetic acid (TFA) and applied to a Pro RPC 5/2 column (Pharmacia, Uppsala, Sweden). A linear gradient elution was performed for 40 min with 0.1% TFA in

acetonitrile. The fraction eluted at 19.4 min representing Lol p 1 was collected, lyophilized and used for further proliferation tests. The purified Lol p 1 allergen was homogeneous as monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Furthermore a contamination of the Lol p 1 preparation with group 5 allergens of rye grass (Lol p 5) was not detectable. The purified Lol p 1 allergen did not contain Lol p 5 impurities as tested by a monoclonal antibody immunoassay (sensitivity of 10 ng/ml for group 5 allergens) for quantitative analysis of group 5 allergens established in our lab [9].

#### Recombinant Allergens

Recombinant Phl p 5b allergens and fragments were cloned, expressed and purified as published elsewhere [7]. The complete recombinant Phl p 5b allergen [rPhl p 5b(1–263)] and two recombinant fragments [rPhl p 5b(1–135), rPhl p 5b(136–263)] were used for proliferation tests.

#### Computer Analysis

Sequence data were analyzed on GeneWorks and PCGENE Software (Intelligenetics, Geel, Belgium) and on DNASIS Software (Pharmacia LKB Biotechnology, Uppsala, Sweden).

#### Patients

Selection of suitable patients (F.R., D.W., C.B., J.R., A.H.) was done as described recently [6]. Three (F.R., D.W., C.B.) of the 5 patients had undergone allergen-specific immunotherapy before establishing TCC.

### Isolation of Phl p 5-Reactive TCC/Proliferation Assays

Phl p 5-reactive TCC were obtained by a technique described recently [6]. Briefly, peripheral blood mononuclear cells (PBMC) were stimulated in 24-well (final volume 1 ml) flat-bottom plates (NUNC, Roskilde, Denmark) using an optimal concentration (5–10 µg/ml) of purified [9] Phl p 5 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).

The Phl p 5-reactive T cell lines obtained were tested regarding the Phl p 5 specificity by a tritiated methylthymidine (<sup>3</sup>H-TdR; Amersham, UK) incorporation assay as described under proliferation assays. 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<sup>4</sup>/well), phytohemagglutinin (1 µg/ml; Biochrom, Berlin, Germany), and IL-2 (25 IU/ml). Before performing allergen-specific proliferation assays, the clones were left to settle for almost 10 days after the last addition of irradiated allogeneic PBMC. Selected Phl p 5-reactive TCC (5×10<sup>5</sup>/well) were expanded in 24-well plates (NUNC; final volume 1 ml) by restimulation with phytohemagglutinin (2.5 µg/ml), irradiated allogeneic feeder cells (5×10<sup>5</sup>/well), and IL-2 (25 IU/ml).

### Proliferation Assays

Initially group 5 reactivities of TCCs were assessed as published recently [6]. After collection of a sufficient panel of Phl p 5-reactive TCCs proliferative responses to recombinant Phl p 5 allergen and fragments as well as to natural Lol p 1 allergen were tested as follows. T cell blasts (in each case 2×10<sup>4</sup> cells/well) were stimulated in triplicate cultures in 96-well round-bottomed plates (NUNC, final volume 0.2 ml) in each case with 10 µg/ml of group 5 (natural or recombinant) and Lol p 1 allergen in the presence of irradiated autologous PBMC (5×10<sup>4</sup>/well) as antigen-presenting cells. After 56 h <sup>3</sup>H-TdR (1 µCi/well) was added for 16 h. Proliferation (<sup>3</sup>H-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 different allergens and the same cultures without allergens) was greater than 10.

## Results

Altogether 51 Phl p 5-reactive TCC (5 from donor F.R., 12 from donor D.W., 11 from donor C.B., 13 from donor J.R. and 10 from donor A.H.) were tested with regard to their proliferative response to complete recombinant Phl p 5b(1–263) and purified Lol p 1. In previous studies the useful (optimal) allergen dose of 10 µg/ml has been established. Appropriate dose-response curves of two representative TCCs are shown in figure 1. As expected all 51 TCC were triggered by the whole recombinant Phl p 5b allergen comparable to the reactivity to natural Phl p 5. Interestingly from each donor

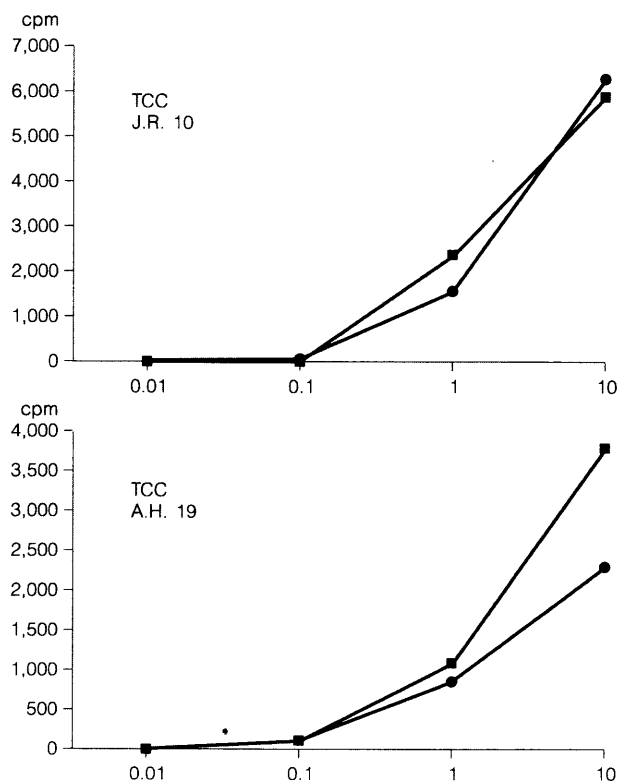


**Fig. 1.** Dose-response curves in two representative Phl p 5/Lol p 1-reactive T cell clones. 2×10<sup>4</sup> cloned T cells were cultured with 5×10<sup>4</sup> irradiated autologous PBMC and different concentrations (0.01–10 µg/ml) of rPhl p 5b(1–263; ■) or natural Lol p 1 (●). The response in the absence of allergen was 90 cpm in J.R. 10 and 70 cpm in A.H. 19. The values shown are the arithmetic mean cpm in triplicate cultures.

Lol p 1 cross-reactive TCC were isolated (see table 1). By means of recombinant Phl p 5b fragments – rPhl p 5b(1–135)/rPhl p 5b(136–263) – two parts of Phl p 5 were tested (see table 1) bearing potential group 5/1 cross-reacting T cell epitopes. Obviously essential epitopes responsible for cross-stimulation by Lol p 1 allergens are located especially within the amino acid residues 1 and 135 of Phl p 5b.

## Discussion

A variable percentage of Phl p 5-reactive TCC (donor F.R.: 2/5; donor D.W.: 1/12; donor C.B.: 2/11; donor J.R.: 6/13; donor A.H.: 4/10) recognizes interspecies T cell epitopes. 'Pseudo cross-reactivities' due to group 5 (Lol p 5) impurities of the Lol p 1 preparation appear to be unlikely as only 15 of 51 TCC tested were cross-stimulated. A contamination of the Lol p 1 preparation used with the Lol p 5 al-

**Table 1.** Proliferative responses of group 5/1 reactive TCC from 5 donors

| TCC  |    | <sup>3</sup> H-TdR, cpm × 10 <sup>3</sup> |         |         |                   |                   |                     |
|------|----|-------------------------------------------|---------|---------|-------------------|-------------------|---------------------|
|      |    | negative control <sup>1</sup>             | Lol p 1 | Phl p 5 | rPhl p 5b (1–263) | rPhl p 5b (1–135) | rPhl p 5b (136–263) |
| D.W. | 28 | 0.10                                      | 1.10    | 6.00    | 4.24              | 1.16              | 0.18                |
| F.R. | 2  | 0.06                                      | 2.43    | 3.66    | 3.00              | 0.83              | 0.05                |
| F.R. | 7  | 0.10                                      | 2.79    | 3.30    | 1.24              | 0.17              | 0.06                |
| C.B. | 3  | 0.12                                      | 1.30    | 3.45    | 3.50              | 1.00              | 0.20                |
| C.B. | 7  | 0.16                                      | 1.70    | 1.66    | 1.79              | 1.32              | 0.17                |
| J.R. | 6  | 0.10                                      | 3.75    | 3.65    | 4.17              | 0.32              | 0.25                |
| J.R. | 7  | 0.06                                      | 5.90    | 6.46    | 5.57              | 4.10              | 0.08                |
| J.R. | 9  | 0.07                                      | 3.53    | 3.64    | 3.32              | 1.35              | 0.13                |
| J.R. | 10 | 0.08                                      | 7.10    | 6.57    | 5.90              | 4.00              | 0.24                |
| J.R. | 15 | 0.07                                      | 7.90    | 6.50    | 5.15              | 2.26              | 0.21                |
| J.R. | 19 | 0.08                                      | 3.95    | 3.00    | 3.12              | 0.84              | 0.06                |
| A.H. | 13 | 0.02                                      | 1.28    | 1.52    | 2.12              | 1.35              | 0.05                |
| A.H. | 19 | 0.05                                      | 2.60    | 4.05    | 5.00              | 1.02              | 0.06                |
| A.H. | 21 | 0.04                                      | 0.60    | 1.77    | 2.68              | 1.58              | 0.05                |
| A.H. | 32 | 0.04                                      | 0.84    | 1.07    | 1.66              | 0.03              | 0.05                |

For each clone the incorporation of <sup>3</sup>H-TdR (Amersham, UK) after stimulation with 10 µg/ml of natural or recombinant allergens is listed. Positive responses are given in bold print (stimulation index > 10). The standard error of the mean of all experiments was < 20%. Data were obtained from several comparable experiments.

<sup>1</sup> Irradiated autologous antigen-presenting cells plus TCC in the absence of allergens.

|        |                                                    |     |
|--------|----------------------------------------------------|-----|
| PHLP5b | ADAGYAPATPA-----AAG-----A-AAGKATTEEOKLIEDINVGF-    | 35  |
| LOL P1 | MASSSSVLLVVALFAVFLGSAHGIARVPPGPNITAEYGDKWLDKASTWYG | 50  |
|        | <i>S S S S S S S S S S S S S S S</i>               |     |
|        |                                                    |     |
| PHLP5b | KAAVAARQR-PAADKFKTFEASPRHPRPL-----RQGA-----G       | 69  |
| LOL P1 | KPTGAGPKDNGGACGYKNVDKAPFNGMTGCGNTPIFKDGRCGSC-----  | 95  |
|        | <i>*SS *S * S* *</i>                               |     |
|        |                                                    |     |
| PHLP5b | LVPKLDAAYSVAYKAAVGATPEAKFDSFVASLTEALRVIAGALEVHAVKP | 119 |
| LOL P1 | -----FEIKCTKPESCSGEAVTVTITDDNEEPIAPYHFDLSGHAFGS    | 137 |
|        | <i>SSS SS S S S *S SSS S* S S*S * S</i>            |     |
|        |                                                    |     |
| PHLP5b | VTEEPGMAKI-PAGELQI-IDKIDAAFKVAATAAATAPADDKFTVFEEAF | 167 |
| LOL P1 | MAKKGEEQNVRSALELEQFRRVKCKY-----PDDTK-----PTF       | 172 |
|        | <i>SSS S S****SS SSS S *S*S* S*</i>                |     |
|        |                                                    |     |
| PHLP5b | NKAIKESTGGAYDTY--KCIPSLAAVKQAYAAATVAAAEEVKYAVFEAAL | 215 |
| LOL P1 | H--VEKASNPNYLAIVKYVDGDGVV----AVDIKEKGDKWIELKESW    | 216 |
|        | <i>S SS SS S* S * S SSS* *SSS S S *S SSSS</i>      |     |
|        |                                                    |     |
| PHLP5b | TKAITAMSEVQKVSQPATGAATVAAGAATTAAGAASGAATVAAGGYKV   | 263 |
| LOL P1 | G-AVWRIDTPDKLTGPFVRYTTEGGTKSEFEDVIPEGWKADTSYSAK    | 263 |
|        | <i>*S SS S* * S * S S SSS SSSSS</i>                |     |

**Fig. 2.** Sequence alignment of the Phl p 5b allergen [7] with the Lol p 1 allergen [12]. Numbers indicate amino acid residues (international one-letter code). Dashes represent gaps in the sequences produced by the aligning program. Perfectly (\*) and well (s) conserved positions in the alignment are marked. Both arrows (position 135/136) distinguish the recombinant fragments tested: rPhl p 5b(1–135) and rPhl p 5b(136–163).

lergen was not detectable by means of our very sensitive monoclonal antibody immunoassay [9].

Unspecific mitogenic effects of the recombinant antigen preparations are impossible too. Among the 51 TCC another 18 clones were not stimulated by fragment rPhl p 5b(1–135), but by fragment rPhl p 5b(136–263). TCCs in particular seem to interact with common epitopes/motifs found on group 5 allergens only. The existence of intraspecies T cell epitopes was demonstrated first, namely in Lol p 1, 2, 3 [4]. Recently interspecies cross-reactivities have also been described [5].

But unlike here Mohapatra et al. [5] tested Lol p 1-reactive TCC for secondary cross-stimulations with recombinant group 5 allergens of Kentucky bluegrass (Poa p 5). We, however, looked for secondary group 1 (Lol p 1) reactivities of group 5 (Phl p 5)-reactive TCC. Our data confirm that both main groups of grass pollen allergens share cross-reacting T cell motifs.

The high degree (90%) of sequence homologies of Phl p 5 to Poa p 5 [11] could be the background for similar data found with recombinant Poa p 5 and natural Lol p 1 allergens [5]. Obviously such cross-sensitizations are no rarity and exist independent of an ongoing immunotherapy (see TCCs from donor J.R. and A.H.). A sequence comparison (see fig. 2) of Lol p 1 [received from 12] and Phl p 5b [7] showed that both allergens possess structural identities of about 10% as well as similarities of about 34%. Especially a

common motif 'AGEL' discussed already by Mohapatra et al. [5] could be of interest for the cross-stimulation found. Interestingly fragment rPhl p 5b(1–135) also bears the latter motif (residues 131–134).

In our experiments it was generally noticed that all 15 TCC tested were stimulated by fragment rPhl p 5b(1–135) with the exception of three TCC (F.R. 7; J.R. 6; A.H. 32). Possibly this TCC recognizes a motif ('PAGEL' enclosed?) located around the contact region of both Phl p 5b fragments. Fragment rPhl p 5b(136–263) turned out to be non-stimulatory for all group 5/1-reactive 15 TCC.

Whether other stretches of residues should be taken into consideration for cross-reactivities to Lol p 1 should be established. Therefore appropriate investigations using overlapping dodecapeptides (offset by 3 residues and overlapping by 9 residues) representing amino acid residues 1–145 of Phl p 5b are starting now.

Elucidation of such epitopes responsible for the interspecies cross-reactivities of major grass pollen allergens is of importance because it could restrict the number of peptides necessary for inclusion in a scientifically based formulation for specific immunotherapy [13].

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### References

- 1 WHO/IUS Allergen Nomenclature Subcommittee World Health Organization, Geneva, Switzerland: Allergen nomenclature. Clin Exp Allergy 1995;25:27–37.
- 2 Matthiesen F, Lowenstein H: Group V allergens in grass pollens. I. Purification and characterization of the group V allergen from *Phleum pratense* pollen, *PhlpV*. Clin Exp Allergy 1991; 21:297–307.
- 3 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.
- 4 Baskar S, Parronchi P, Mohapatra S, Romagnani S, Ansari AA: Human T cell responses to purified pollen allergens of the grass, *Lolium perenne*. Analysis of relationship between structural homology and T cell recognition. J Immunol 1992;148:2378–2383.
- 5 Mohapatra SS, Mohapatra S, Yang M, Ansari AA, Parronchi P, Maggi E, Romagnani S: Molecular basis of cross-reactivity among allergen-specific human T cells: T cell receptor V gene usage and epitope structure. Immunology 1994;81:15–20.
- 6 Müller WD, Karamfilov T, Fahlbusch B, Vogel-sang H, Jäger L: Analysis of human T cell clones reactive with group V grass pollen allergens. Int Arch Allergy Immunol 1994;105: 391–396.
- 7 Bufer A, Schramm G, Keown MB, Schlaak M, Becker WM: Major allergen *PhlpVb* in timothy grass is a novel RNase. FEBS Lett 1995;363: 6–12.
- 8 Fahlbusch B, Müller WD, Diener C, Jäger L: Detection of cross-reactive determinants in grass pollen extracts using monoclonal antibodies against group IV and V allergens. Clin Exp Allergy 1993;23:51–60.
- 9 Fahlbusch B, Müller WD, Schlenvoigt G, Jäger L, Wahl R, Weber B: Monoclonal antibody immunoassay for quantitative analysis of group V allergens in grass pollen extracts. Clin Exp Allergy 1993;23:747–754.
- 10 Fahlbusch B, Müller WD, Cromwell O, Jäger L: Application of reversed-phase high-performance liquid chromatography for purification of major allergens from grass pollen. J Immunol Methods, submitted.
- 11 Vrtala S, Sperr WR, Reimnitzer J, Van Ree R, Laffer S, Müller WD, Valent P, Lechner K, Rumpold H, Kraft D, Scheiner O, Valenta R: cDNA cloning of a major allergen from timothy grass (*Phleum pratense*) pollen: Characterization of the recombinant *PhlpV* allergen. J Immunol 1993;151:4773–4781.
- 12 Perez M, Ishioka GY, Walker LE, Chesnut RW: cDNA cloning and immunological characterization of the rye grass allergen Lol pI. J Biol Chem 1990;265:16210–16215.
- 13 Hetzel Ch, Lamb J: Short analytical review: CD4+ T-cell-targeted immunomodulation and the therapy of allergic diseases. Clin Immunol Immunopathol 1994;73:1–10.