

Immunological changes during specific immunotherapy of grass pollen allergy: reduced lymphoproliferative responses to allergen and shift from TH₂ to TH₁ in T-cell clones specific for Phl p 1, a major grass pollen allergen

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Summary

Background and Objective The mechanisms operative in specific immunotherapy (SIT) of Type I allergy are not completely understood. In the present study we evaluated immunological changes during SIT in pollinosis.

Method Eight patients suffering from pollinosis (monosensitized to grass pollen) were treated with conventional SIT. All subjects had IgE specific for Phl p 1, a major allergen of timothy grass. *In vitro* changes in the immunological reactivity to grass pollen extract and to recombinant Phl p 1 were evaluated. Subjects were examined at three occasions: before, after 3 months and after 1 year of SIT.

Results Serological analysis revealed a marked increase of grass pollen- and Phl p 1-specific IgG, titres of specific IgE did not change significantly. Lymphoproliferative responses to grass pollen extract and rPhl p 1 were reduced already after 3 months of treatment. Accordingly, the cloning efficiency for Phl p 1-specific T-cell clones (TCC) dropped markedly in all patients. The majority of allergen-specific TCC raised before SIT revealed a TH₂-like pattern of cytokine production, TCC established after SIT revealed TH₁ characteristics. This shift was due to a decrease in IL-4 rather than an increase in IFN- γ production by T cells. Investigations of the epitopes recognized by T cells before and after SIT did not reveal the outgrowth of new ('protecting') specificities. We could not observe induction of allergen-specific CD8⁺ lymphocytes (suppressor cells).

Conclusion Our data indicate that — on the level of TH lymphocytes — SIT induces tolerance to the allergen and a modulation of the cytokine pattern produced in response to allergen stimulation.

Keywords: allergy, immunotherapy, TH₁, TH₂

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Introduction

Numerous controlled clinical trials have demonstrated the efficacy of specific immunotherapy (SIT) [1] in selected patients suffering from Type I allergy [2–4]. The mechanisms operative in successful immunotherapy, however, are

still poorly understood. SIT leads to a rise of allergen-specific IgG, which had previously been interpreted as the formation of 'blocking antibodies' [5], however, identical IgG-rises were observed in non-responders to SIT [6–10]. Other authors described a reduction in mast cells and/or eosinophils and the associated mediators as a consequence of successful SIT [11–13]. Moreover, the generation of antigen-specific suppressor cells has been proposed [14]. In the last few years, a pathway of IgE-regulation, essentially

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based on the reciprocal activities of IL-4 and IFN γ was discovered [15–17]. It could be shown that T-cell clones (TCC) established from atopic individuals, with specificity for allergens could characteristically be attributed to a TH₂-like subset (high production of IL-4 and IL-5), whereas TCC specific for bacterial antigens produced IFN γ upon stimulation, thus belonging to the TH₁-like subset [18]. Furthermore, lymphocytes in typical allergic inflammation sites could characteristically be attributed to the TH₂ subset [19,20]. Recent publications indicated a shift from a more TH₂-like type of immune response to TH₁ after successful SIT [21–24]. In one retrospective study it was shown that allergen-specific TCC raised from patients who had undergone SIT produced less IL-4 when compared with allergen-specific TH cells from non-treated house dust mite- and grass pollen-allergic subjects [21]. A decrease of IL-4 and IL-5 and an increase of IFN γ was observed in specific T cells after SIT with bee venom [22]. In another approach — analysing allergen-induced cellular infiltrations in the skin and the nose — it was shown that successful SIT inhibited early- and late-phase responses and was accompanied by local decreases in activated T lymphocytes, and these T cells moreover revealed TH₁ characteristics [23–24].

In this study we evaluated the immune response to *Phleum pratense* and to recombinant Phl p 1 [25,26], a major allergen of timothy grass in patients suffering from grass pollen allergy during the course of SIT. Humoral and cellular reactivities were measured and evaluated before SIT, after the 3-months dose increase phase and after 1 year of treatment. We hypothesized that allergen-reactive T cells would either be impaired by the treatment (tolerance induction) or undergo a modulation from a TH₂-like phenotype to TH₁/TH₀ (immune deviation). Moreover, epitope mapping studies were performed in order to observe shifts in the pattern of epitope recognition by these T lymphocytes after SIT.

Materials and methods

Patients and study design

Eight patients (three female, five male; mean age 26.5 years) were included in the study. All patients were mono-sensitized to grass pollen and displayed typical clinical symptoms during the pollen season. Specific IgE to grass pollen was measured by RAST, the presence of specific IgE to Phl p 1 was proved by ELISA. All patients displayed positive skin-prick tests to grass pollen extract. HLA-typing was performed and revealed: individual 1: DRB1*01/03, DRB3, DQB1*01/02; 2: DRB1*03/07, DRB3, DRB4, DQB1*02/03; 3: DRB1*03/07, DRB3, DRB4, DQB1*02/03; 4: DRB1*03/16, DRB3, DRB5, DQB1*01/02; 5: DRB1*04/13, DRB3, DRB4, DQB1*03/03; 6: DRB1*13/

14, DRB3, DQB1*01; 7: DRB1*01/04, DRB4, DQB1*01/03; 8: ND.

SIT was performed using grass pollen extract (Alutard SQ 200) obtained from ALK, Horsholm, Denmark. Immunotherapy was performed according to international standards using a conventional immunotherapy protocol [27]. The clinical and overall constellation of the patients represented an indication for SIT [4]. Clinical success of SIT was evaluated by subjective case history, verified by symptom- and medication score calendars [9].

All evaluations were performed in an identical way at three occasions: before SIT (September; phase I), after the 3 month period of dose-increase with weekly injections (December/January; phase II), after 1 year treatment with the maintenance dose (monthly injections; September/October, phase III).

Cellular tests included: proliferation of PBMC to grass pollen extract and r Phl p 1, establishment of Phl p 1-specific TCL and TCC, characterization of cytokine profile and epitope mapping of Phl p 1-specific TCL and TCC.

Antibody determinations: IgE-immunoblot analysis using complete grass pollen extract were performed. R Phl p 1-specific IgE, IgG and IgG1 and IgG4 were measured by ELISA.

Allergens

Pollen from *Phleum pratense* was obtained from Allergon (Engelholm, Sweden). Complete grass pollen extract was produced as described. RPhl p 1 was expressed as non-fusion protein and purified as described [28].

Antibody measurements

ELISA: Microtitre plates (M129 A, Dynatech MT, Zug, Switzerland) were coated with rPhl p 1 (2 μ g/mL in PBS), thereafter ELISA was performed as described [29] using mouse-anti-human IgG, IgG1, IgG4 and IgE antibodies (Pharmingen, San Diego, CA, USA) and sheep-anti-mouse Ig-POX labelled antibodies (Amersham, Buckinghamshire, UK) for detection. Sera were titrated starting from an 1:50 dilution to 1:31650 in case of IgG and IgG subclasses, and 1:2–1:32 for IgE-determination. Immunoblots with complete extract of *Phleum pratense* were performed as described [26]. In order to guarantee comparable results, sera were kept frozen until the end of the study and all antibody determinations were performed in one experiment.

Proliferation assays

All proliferation assays were performed in triplicates, incubation periods before addition of [³H] TdR were 6 days

for PBMC, and 48 h for TCL and TCC, respectively [30]. Lymphoproliferative responses to complete grass pollen extract and to rPhl p 1 were measured for six concentrations from 25 µg/mL to 0.75 µg/mL.

Allergen-specific T-cell lines

Allergen-specific TCL were obtained by a technique described [30]. Briefly, 0.5×10^6 PBMC were stimulated with individual optimal concentrations (5–20 µg/mL) of purified rPhl p 1 in Ultra Culture Medium (Bio Whittaker, Walkersville, MD, USA) supplemented with 2 mM/L glutamine and 2×10^{-5} M 2-ME in 24-well flat-bottomed culture plates (3524 Costar, Cambridge, MA, USA) for 5 days. Subsequently, human r-IL-2 (10 U/mL, Böhringer, Mannheim, Germany) was added and the culture continued for additional 7 days.

Allergen-specific T-cell clones

T-cell blasts from rPhl p 1-specific TCL were seeded in limiting dilution (0.3 cells/well) in 96 well round bottom plates (Nuncclone, Nunc, Denmark) in the presence of 10^5 irradiated (5000 rad) allogeneic PBMC as 'feeder cells', 1% PHA v/v (Gibco, Grand Island, NY, USA) and rIL-2 (4 U/well) in the above described medium. Growing microcultures were then expanded at weekly intervals with fresh feeder cells and rIL-2. The specificity of TCC was assessed as previously described [26].

Epitope mapping

T-cell epitope mapping was performed with Phl p 1-specific TCL and TCC, using 77 peptides (Cambridge Research Biochemicals Limited, Cambridge, UK) overlapping by nine amino acids and spanning the whole sequence of the protein as described [26]. Briefly, Phl p 1-TCL were directly tested with all 77 peptides, and SI > 5 were considered as positive. TCC reacting with Phl p 1 (stimulation index > 1.0) were tested with seven pools containing 11 peptides, the amount of each peptide in the test well 1 µg (5 µg/mL). In a second series, the positive pool (SI > 10) was split up, and the TCC cells were retested with the peptides separately in duplicates (5 µg/mL).

Analysis of the phenotype of TCC

Antibodies: FITC-labelled MoAb anti-Leu 4/CD3, anti-Leu 3a/CD4, anti-Leu 2a/CD8, anti-T cell antigen receptor TCR-1 $\alpha\beta$ (WT31 and anti-TCR $\gamma\delta$ (I (Becton Dickinson, Mountain View, CA, USA) were used to analyse the phenotype of the TCC. Flow cytometry analysis was performed as described [30].

Stimulation for cytokine production

10^6 cells from TCL or TCC were washed and incubated with 10^6 irradiated autologous APC in the presence of Phl p 1 (10 µg/mL) in 1 mL medium. Supernatants (SN) from allergen-stimulated cultures were collected after 24 h of incubation [30].

Cytokine assays

IL-4 levels in the SN were measured using a sandwich ELISA technique (Quantikine; R&D Systems, Minneapolis, MN, USA), sensitivity threshold: 3 pg/mL; IFN γ was determined by solid-phase ELISA, sensitivity threshold: 1.5 pg/mL (Medgenix Diagnostix, Fleurus, The Netherlands). SN collected from cultures containing allergen and irradiated feeder cells alone were used as negative controls. For each individual, values of IL-4 and IFN γ production of all allergen-specific TCC established before and after commencement of SIT were compared using Mann-Whitney U-test.

Results

Clinical success of SIT

Seven out of eight patients displayed a marked improvement of their allergy symptoms during the first grass pollen season under SIT. One individual (subject 8, Table 1, Figs 1 & 2) only experienced a slight decrease of allergy symptoms. The subjective data supplied by the patients correlated well with a reduced need of anti-allergic medication.

Antibody determinations

ELISA experiments showed a marked rise in Phl p 1-specific IgG, IgG1 and IgG4 in all patients already after the dose increase phase (phase II) and a further rise in IgG

Table 1. Number of Phl p 1-specific TCC established by an identical protocol before (I), after the dose increase phase (II) and after 1 year of SIT (III)

TCC	Patients								Total	Mean
	1	2	3	4	5	6	7	8		
I	16	14	24	13	27	12	5	5	116	14.5
II	8	5	2	11	5	5	ND	3	39	5.6
III	2	3	1	ND	5	3	2	4	20	2.9
	26	22	27	24	37	20	7	12	175	

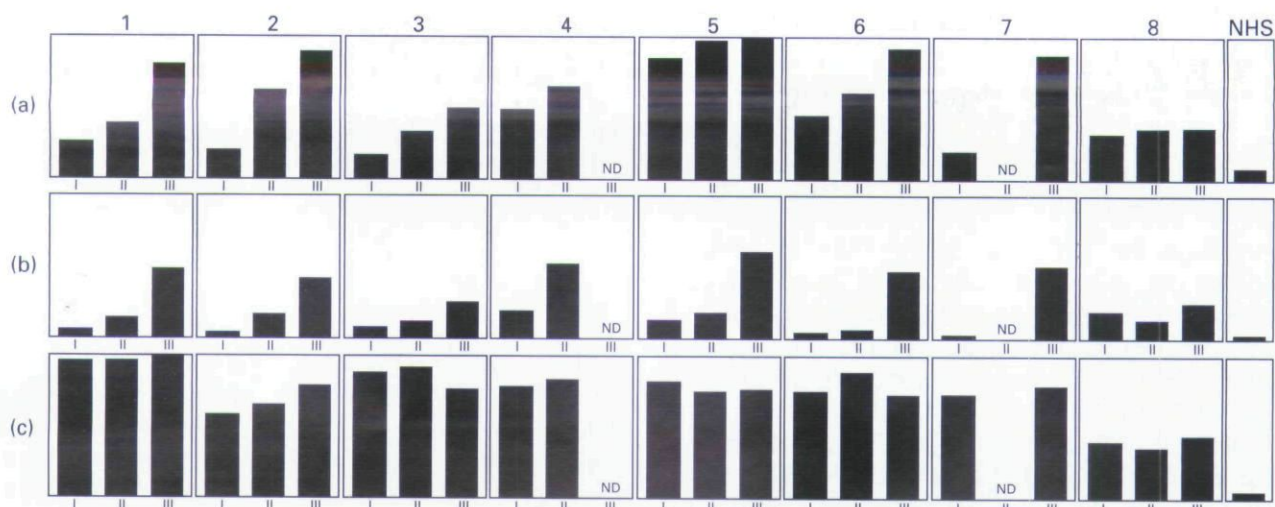


Fig. 1. Changes in serum-Phl p 1-specific IgG, IgG4 and IgE during SIT. I, before SIT; II, after the dose increase phase; III, after 1 year of SIT. (a) Total IgG; (b) IgG4; (c) IgE, bars represent % absorption.

after 1 year of treatment (phase III; Fig. 1). IgE levels did not change significantly in the course of therapy (Fig. 1). Immunoblots showed no changes in intensity and quality of IgE-binding to allergens in complete grass pollen extract (Fig. 2).

Proliferation assays and T-cell cloning efficiency

A significant decrease in the lymphoproliferative responses to grass pollen extract and to rPhl p 1 could be observed already after phase II (dose increase phase), this effect was also visible after 1 year of SIT (phase III; Fig. 3). This picture was reflected by a drastic decrease of cloning efficiency for allergen-specific T cells in the course of SIT. In total, 175 Phl p 1-specific TCC were produced and tested. The recovery of Phl p 1-specific TCC before SIT was 116 (mean: 14.5/patient), after phase II 39 Phl p 1 specific TCC could be established from TCLs (mean: 5.6) and after

phase III, using the identical protocol, 20 Phl p 1-specific TCC (mean 2.9) could be isolated from short-term TCL (Table 1).

Analysis of phenotype of TCC

In total 175 TCC were analysed. One hundred and seventy-three of 175 (98.9%) revealed the TH phenotype ($CD3^+$, $CD4^+$, $CD8^-$), 2/175 (1.1%) were $CD8^+$, both derived from donor 4 before SIT. All TCC expressed $\alpha\beta$ (TCR). Before SIT, 60/116 (51.7%) of TCC revealed a TH₂-like pattern of cytokine production ($IL4/IFN\gamma > 10$; w/w), 37/31.9% TCC could be classified as TH₀-like ($IL4/IFN\gamma = 10-0.1$) and 19/16.3% were TH₁ ($IL4/IFN\gamma < 0.1$). After phase II, the ratio of TH₂:TH₀:TH₁ was 41:41:18 and after 1 year of treatment (phase III) 3/20 (15%) of TCC displayed TH₂ characteristics, 5/20 (25%) produced IL-4 and IFN γ (TH₀), and 12/20 (60%) were of

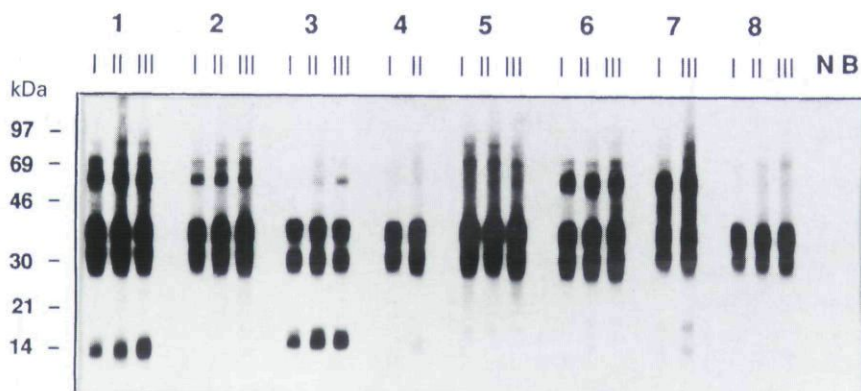


Fig. 2. IgE-immunoblot: serum-IgE reactivity to allergens in complete timothy grass pollen extract during SIT. No changes in IgE-binding patterns to grass pollen allergens can be observed. I, before SIT; II, after the dose increase phase; III, after 1 year of SIT.

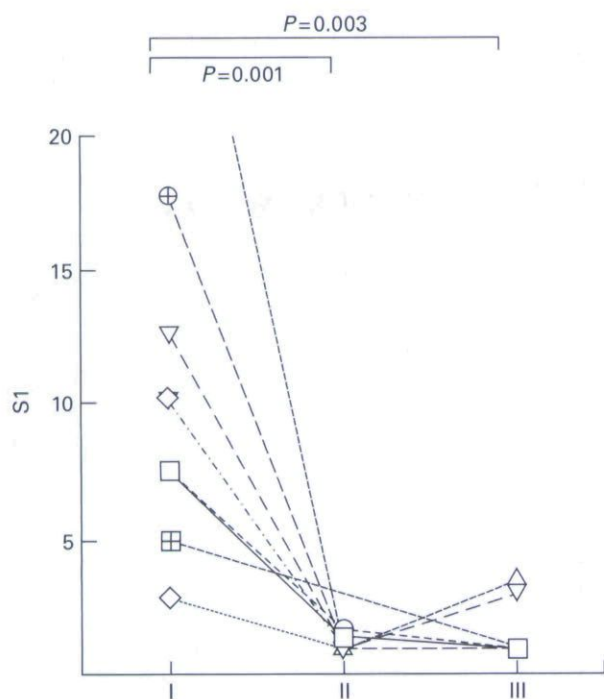


Fig. 3. Proliferation assays using freshly isolated PBMC incubated with recombinant Phl p 1. The allergen concentration individually inducing best proliferation before SIT (5–25 $\mu\text{g}/\text{mL}$) was used throughout the study. Proliferation was significantly reduced already after the phase of dose increase (II). I: before SIT, III: after 1 year of SIT.

the TH₁-like type (Fig. 4). In all patients, IL-4 levels produced by TCC established before commencement of SIT were higher than those of TCC made after induction of SIT. In two out of eight patients this difference reached the level of statistical significance ($P < 0.05$, Mann–

Whitney *U*-test). In contrast, the levels of IFN γ increased in two out of eight patients, decreased in two out of eight and were comparable in four out of eight patients analysed.

T-cell epitopes

All TCL and 138 TCC were epitope mapped. In total 19 T-cell epitopes were detected. The majority of TCC reacted with peptides located at positions on the molecule which have previously been described [26], however, four new T-cell activating regions were identified (amino acid positions: Phl p 1 7–24, 43–63, 58–72, 181–192). Epitope recognition patterns did not reveal the outgrowth of dominant new specificities associated with SIT (Fig. 5).

Discussion

During the last years the crucial role of CD4⁺ TH cells in the induction and maintenance of atopic allergy has been recognized [16–21]. According to recent literature, a SIT-induced modulation of the immune response to allergens at the level of helper T cells can be assumed as working mechanism of the treatment [21–24]. In the present study, eight grass pollen-allergic individuals were followed-up for 1 year of SIT. All patients were monosensitized to grass pollen, young (mean = 26.5 years), and suffering from strictly seasonally limited symptoms during the grass pollen season [5]. Patients responded to the therapy, i.e. they experienced an improvement of their allergy symptoms during the following grass pollen season. Blood was collected at three occasions and analysed for the course of specific antibodies and lymphoproliferative response to grass pollen extract and a well defined recombinant allergen: Phl p 1, an important major allergen of timothy grass. Moreover, Phl p 1-TCL and TCC were produced and

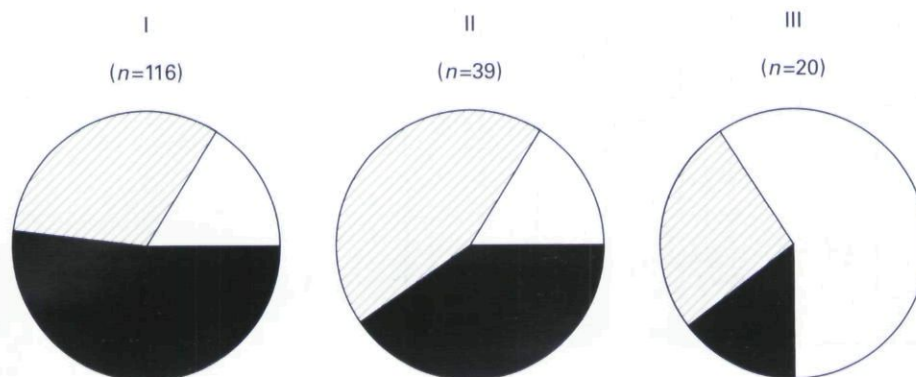


Fig. 4. Ratio of TH₂ vs TH₀ and TH₁ TCC: 10^6 T cells were stimulated with 10 $\mu\text{g}/\text{mL}$ rPhl p 1 in the presence of 10^6 autologous APC in 1 mL medium. Supernatants were collected after 24 h of incubation and tested for IL-4 and IFN γ content by specific ELISA. Before SIT (I), a predominance of TH₂-like TCC can be observed, whereas after 1 year of SIT TH₁-like TCC dominate. II: after the phase of dose increase. ■ TH₂, ▨ TH₁, □ TH₀.

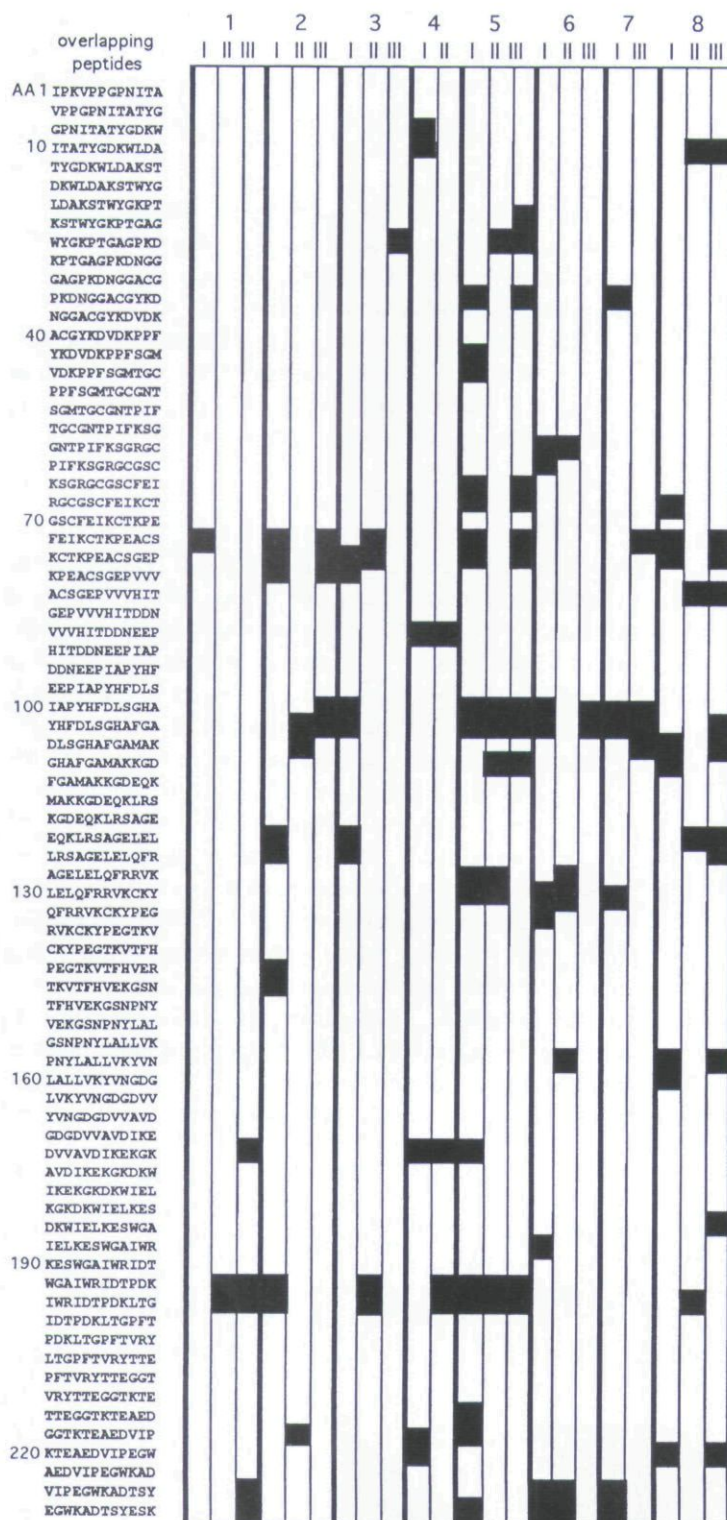


Fig. 5. Ph1 p 1 T-cell epitopes recognized by Ph1 p 1-specific TCL and TCC. I: before SIT; II: after the phase of dose increase; III: after 1 year of SIT. Each black mark represents a peptide fulfilling following criteria: Induction of proliferation (SI > 5) in a Ph1 p 1-T-cell line and/or SI > 10 in Ph1 p 1-specific TCC.

* HLA-DRB and DQB haplotypes of individual 1: DRB1*01/03, DRB3, DQB1*01/02; 2: DRB1*03/07, DRB3, DRB4, DQB1*02/03; 3: DRB1*03/07, DRB3, DRB4, DQB1*02/03; 4: DRB1*03/16, DRB3, DRB5, DQB1*01/02; 5: DRB1*04/13, DRB3, DRB4, DQB1*03/03; 6: DRB1*13/14, DRB3, DQB1*01; 7: DRB1*01/04, DRB4, DQB1*01/03; 8: ND.

analysed for cytokine production in response to specific stimulation and for epitope recognition.

As expected, we could observe the SIT-characteristic changes in antibody composition: a rise in specific IgG (predominantly IgG4) whereas specific IgE did not change quantitatively nor qualitatively (Figs 1 & 2). We found a marked decrease in the lymphoproliferative response of PBMC to timothy grass pollen extract and to Phl p 1 already 3 months after the commencement of SIT, when the phase of dose-increase was terminated (Fig. 3) [31–33]. Secrist *et al.* reported significant proliferation to allergens after SIT, however, in this study the patients were not compared with themselves at different time points of SIT, but with a non-treated control group [21]. Flow cytometry analyses of the CD4/CD8 ratio in allergen-stimulated T-cell cultures displayed no significant changes in the composition of these phenotypes in TCLs in the course of therapy (not shown). As could be expected from our proliferation assays, the cloning efficiency for Phl p 1-specific TCC from allergen-stimulated short-term TCLs decreased (Table 1). Though identical protocols were used, the yield of allergen-specific TCC of our patients before treatment was 116, after 1 year of SIT only 20 Phl p 1-specific clones could be established from the donors. This marked decrease in allergen-specific T-cell reactivity reflects the induction of tolerance to the injected allergen. In our hands, no induction of allergen-specific CD8⁺ suppressors [15,33], neither in TCL nor in the TCC derived thereof became evident. However, the induction of suppressor cells [34,35] which are not allergen-reactive cannot be excluded, since we only selected for Phl p 1-specific T cells. The analysis of cytokine production patterns (IL4/IFN γ) in SN did not lead to conclusive results in TCL, obviously due to the low ratio ($\approx$ 1:100–1:1000) of allergen-reactive T lymphocytes in these cultures. However, analysis of Phl p 1-specific TCC from allergic donors before, after 3 months, and after 1 year of SIT showed a continuous shift from a predominant TH₂/TH₀ phenotype before to a TH₁/TH₀ pattern of cytokine production after SIT (Fig. 4). The immune deviation observed can [in accordance with Secrist *et al.* 21] be assigned to a decrease in IL-4-production by allergen-specific TCC which was visible in all our patients. However, due to low case numbers, statistical significance could only be calculated for two patients. In some cases the drop in IL-4 was accompanied by an increase of IFN γ . However, IFN γ levels stayed constant or even decreased in other individuals [21,22]. Previous reports indicated an increase of IFN γ -producing cells in allergen-induced infiltrates of skin and nasal mucosa [23,24]. One possible explanation for the discrepancy may be the different repertoire of lymphocytes in these tissues as compared with the peripheral blood.

Epitope-mapping studies using overlapping peptides representing the whole Phl p 1 sequence were performed.

In total, 19 regions with T-cell activating capacity could be identified (Fig. 5). T-cell recognition displayed broad inter-individual diversity [36–42], however, the T-cell activating fragments of Phl p 1 described recently to represent 'major epitopes' [26] were also recognized in this patients collective (displaying different HLA types). Most epitope specificities found before therapy could also be seen in the further course of the treatment, i.e. no clear SIT-associated changes in the individual T-cell recognition patterns could be detected. These data could mean that SIT leads to a modulation of the TH response in currently existing, relevant allergen-specific TCC rather than to an expansion of a 'new' population of T cells.

Since this study included a low number of patients, which all improved during SIT, we can only presume that the observed mechanisms correlate with the clinical success of SIT and it remains to be studied whether non-responders to SIT lack similar changes in immunoreactivity to the injected allergen. However, our *in vitro* data suggest that conventional injection immunotherapy leads to a modulation of the immune response to the allergen on the level of TH cells, which may even be specific for TH₂ lymphocytes. In synopsis with published work, we encourage the development of new strategies of SIT targeted on allergen-specific T cells [43,44].

Acknowledgments

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