

ORIGINAL PAPER

Absence of systemic immunologic changes during dose build-up phase and early maintenance period in effective specific sublingual immunotherapy in children

E. Dehlink*, T. Eiwegger*, M. Gerstmayr*, E. Kamp†, B. Bohle†, K.-W. Chen†, S. Vrtala†, R. Urbanek* and Z. Szépfalusi*

*Department of Pediatrics and Juvenile Medicine and †Department of Pathophysiology, Centre for Physiology and Pathophysiology, Medical University of Vienna, Vienna, Austria

Clinical and Experimental Allergy

Summary

Background Sublingual immunotherapy (SLIT) has been reported to be a safe treatment for inhalant allergies in children. Yet the immunologic mechanisms resulting in clinical improvement are poorly understood.

Objective To identify early systemic immunologic changes during the first 8 weeks of clinically effective SLIT to grass pollen, tree pollen or house dust mite in paediatric patients with allergic rhinoconjunctivitis and/or asthma.

Methods Peripheral blood mononuclear cells and plasma samples of 13 children with reduced symptoms after 1 year of SLIT were obtained before therapy and at 2 and 8 weeks after the initiation of SLIT. Allergen-specific lymphocyte proliferation assays were performed, and allergen-induced cytokine production (IL-2, IL-4, IL-10, IFN- γ , and TGF- β_1) was measured by ELISA and flow cytometry. Allergen-specific IgE, IgG1, IgG4, and IgA levels in plasma samples were determined in ELISA.

Results During the first 8 weeks of successful SLIT, allergen-specific lymphoproliferation ($n = 13$) as well as levels of allergen-specific intracellular ($n = 8$) and secreted cytokines ($n = 9$) did not change significantly. In addition, no alterations in levels of allergen-specific Igs ($n = 7$) were observed.

Conclusion We could not find any early systemic immunologic changes during the first 8 weeks of clinically effective SLIT to inhalant allergens in paediatric patients with allergic rhinoconjunctivitis and/or asthma.

Keywords children, cytokines, ELISA, flow cytometry, Igs, lymphocyte proliferation assay, sublingual immunotherapy

Submitted 2 March 2005; revised 3 August 2005; accepted 13 October 2005

Correspondence:

Zsolt Szépfalusi, Department of Pediatrics, Division of General Pediatrics, Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria.
E-mail: zsolt.szepfalusi@meduniwien.ac.at

Introduction

Allergen-specific immunotherapy via the subcutaneous injection route (SIT) has been shown to be effective in the treatment of allergic disorders such as asthma and rhinitis [1], but the risk of severe systemic side-effects and the need for frequent injections limit the application of this therapy, especially in children [2, 3]. Sublingual allergen administration (sublingual immunotherapy (SLIT)) may represent a viable alternative to the parenteral injection treatment [4]. The idea of administering allergens via the mucosal route originates from animal studies indicating a predisposition of mucosal surfaces to generate tolerance after antigenic stimulation [5–7].

A recently published Cochrane Review (1966–2002) revealed a significant reduction in symptoms of allergic rhinitis (AR) and use of medication following SLIT in adults (22 double-blind, placebo-controlled trials, 979 participants). However, no significant effects were seen in studies exclusively involving children (five studies, 218 subjects) [8]. Since 2002, several more studies including children have been performed, and it has been concluded that SLIT is only marginally effective in paediatric patients and should therefore be recommended to selected subpopulations, namely asthmatic children monosensitized to house dust mites (HDMs) [9, 10].

The immunologic mechanisms of SLIT are still unclear: inconsistent alterations in allergen-specific serum IgE and

IgG4 [11–22], reduced lymphoproliferative responses [14], a decrease in eosinophils, and a down-regulation of ICAM-1 expression in the nose [23] and the conjunctiva after specific allergen challenge [24] following SLIT have been observed.

The aim of the present study was to investigate the immunologic mechanisms during the very early phase of SLIT, in particular to potentially identify early alterations in systemic immunologic parameters in clinically effective SLIT in children. For this purpose, cellular (lymphoproliferation and cytokine production of allergen-specific T cells) as well as humoral immunologic parameters (allergen-specific Ig levels) were followed during dose-incremental phase and early maintenance period (first 8 weeks) of successful SLIT for inhalant allergens in paediatric patients suffering from allergic asthma and/or rhinoconjunctivitis.

Materials and methods

Study population and immunotherapy protocol

Thirteen children (seven males, six females, aged 5–13 years, median 9 years) attending the Asthma and Allergy Outpatients' Department at the University Children's Hospital of Vienna, who reported a reduction of clinical symptoms after 1 year of SLIT to either HDM ($n = 9$), grass pollen ($n = 3$), or birch pollen ($n = 1$) were included in this study. SLIT had been prescribed on the basis of clinical symptoms (AR and/or bronchial asthma), a positive skin prick test and/or specific IgE > CAP/RAST class 2 to one of the three inhalant allergens, a minimum age of 5 years, sensitization to a small spectrum of allergens, and good compliance. SLIT was performed according to a standard protocol using standardized allergen extracts of HDMs (*Dermatophagoides pteronyssinus* and *D. farinae*), grass pollen (timothy grass, banyard grass, and ryegrass pollen), or tree pollen (birch, hazel, and alder pollen, all Alk-Abelló, Horsholm, Denmark). Therapy started with a 28-day build-up phase, when daily increasing amounts of allergen had to be taken under the tongue before being swallowed (one to 10 drops of vial 1–4, containing 0.5, 50, and 500 STU/mL of allergen, respectively; STU, specific treatment units), and passed into maintenance period, when 10 drops of vial 4 were administered three times a week for 2 years. According to the manufacturer, the maintenance dose contained 0.8 µg *D. pteronyssinus* 1 (Der p 1) plus *D. farinae* 1 (Der f 1), 0.4 µg *Phleum pratense* 5 (Phl p 5), or 4.4 µg *Betula verrucosa* 1 (Bet v 1). Hence, the monthly administered cumulative doses were 9.6 µg Der p 1 and Der f 1, 4.8 µg Phl p 5, or 52.8 µg Bet v 1. Corresponding doses of the monthly applied subcutaneous maintenance therapy would contain 9.8 µg Der p 1, 20.2 µg Phl p 5, and 12.3 µg Bet v 1, respectively. Treatment compliance was controlled at each study visit

(beginning of SLIT, after 2 weeks, after 8 weeks, and after 1 year of therapy, respectively), and clinical symptoms were evaluated at baseline and after 1 year.

The experimental protocol was approved by the Vienna General Hospital's Ethics committee, and all patients' parents gave informed consent. No side-effects were reported during SLIT.

Study design

Heparinized venous blood samples were collected immediately before the initiation of SLIT, after 2 weeks and after 8 weeks of therapy. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden). Cells were frozen and stored in liquid nitrogen at -180°C . Plasma samples were obtained from supernatants after density gradient centrifugation and stored at -20°C .

Antigens

Affinity-purified HDM allergen 1 (Der p 1) was generously provided by Allergopharma (Reinbeck, Germany). Recombinant timothy grass pollen allergen 1 (rPhl p 1) and recombinant birch pollen allergen 1 (rBet v 1) were purchased from Biomay (Vienna, Austria). Tetanus toxoid antigen was obtained from Calbiochem (San Diego, CA, USA). Contamination of allergen/antigen preparations by endotoxin was excluded using the EndochromTM assay (Carles River Endosafe, Charleston, SC, USA) according to the manufacturer's instructions. Recombinant HDM allergens were provided by Susanne Vrtala (Department of Pathophysiology, Centre for Physiology and Pathophysiology, Medical University of Vienna, Vienna, Austria).

Lymphoproliferation assays

Triplicates of 1×10^5 PBMC/200 µL serum-free Ultra culture complete medium (Ultra culture medium, Bio-whittaker, Walkersville, MD, USA supplemented with 2 mM L-glutamine and 170 mg/L gentamycin sulphate, Sigma Sciences, St. Louis, MO, USA) were co-cultured with 10 µg/mL Der p 1 [25], 10 µg/mL rPhl p 1 [26], 10 µg/mL rBet v 1 [27], or 1 µg/mL tetanus toxoid. Recombinant IL-2 (25 IU/mL, Roche Diagnostics, Basel, Switzerland) served as positive control. Proliferation was measured after 6 days of culture by detecting the incorporation of 3H-Thymidin (Amersham Int., Amersham, Little Chalfont, Buckinghamshire, UK) as described elsewhere [26]. Results are expressed as stimulation index (SI = mean c.p.m of stimulated triplicate cultures/mean c.p.m of unstimulated triplicate cultures).

Induction of allergen-specific cytokine synthesis in peripheral blood mononuclear cells

PBMC were cultured as described earlier [28]. Briefly, 1×10^6 PBMC/mL Ultra culture complete medium were stimulated with either 1% v/v phytohaemagglutinin (PHA, Life Technologies, Grand Island, NY, USA) or PHA plus the corresponding antigen (10 µg/mL Der p 1, 20 µg/mL rPhl p 1, 10 µg/mL rBet v 1, or 1 µg/mL tetanus toxoid). Antigen was re-added on day 3. On days 3 and 7, 20 IU/mL recombinant IL-2 (Roche Diagnostics) was added to each culture. After 12 days, 2×10^6 cells/mL were stimulated for 4 h with 10 ng/mL phorbol myristate acetate, 1.25 µM ionomycin, and 5 µg/mL brefeldin A (all Sigma Sciences) for the detection of intracellular cytokines. To measure released cytokines in supernatants, PBMC were stimulated at a concentration of 1×10^6 cells/mL without brefeldin A.

Detection of intracellular cytokines

Simultaneous staining of surface markers and intracellular cytokines (CD4-FITC, CD8-PerCP, IL-2-PE, IL-4-PE, IFN-γ-PE, all Becton Dickinson, San Jose, CA, USA, IFN-γ-APC, IL-10-APC, PharMingen, San Diego, CA, USA) was performed as described previously [29]. Using a FACSCalibur four-colour cytometer equipped with CellQuestPro software (Becton Dickinson), analysis of cytokine profiles (IL-2, IL-4, IL-10, and IFN-γ) in CD4⁺ and CD8⁺ lymphocytes, respectively, was realized by combining the gate for lymphocytes with a CD4⁺ or CD8⁺ gating window. Results are expressed as percentages of CD4⁺ and CD8⁺ T-cells containing the studied cytokines, respectively.

Detection of secreted cytokines

Levels of IL-4, IL-10, and IFN-γ in supernatants were measured by ELISA using matched antibody pairs (Endogen, Woburn, MA, USA) according to the manufacturers' instructions (sensitivity limits: IL-4, 9 pg/mL; IFN-γ, 9.5 pg/mL; IL-10, 4.7 pg/mL [30]). Transforming growth

factor (TGF) – β_1 levels were detected using a solid phase ELISA-Kit (DRG International Inc., Mountainside, NY, USA) according to the manufacturer's instructions (sensitivity threshold 1.9 ng/L).

Detection of allergen-specific immunoglobulin E, immunoglobulin A, and immunoglobulin G subclasses

IgE, IgA, IgG1, and IgG4 specific for recombinant Der p 1, Der p 2, Der p 5, Der p 7, and Der p extract in plasma were measured by ELISA as described elsewhere [31].

Statistical analysis

Data were analysed using SPSS for windows (SPSS Inc., Chicago, IL, USA). Non-parametric Wilcoxon' matched-pairs signed-rank test was used to compare alterations in cellular and humoral immunologic parameters at the different times of the study. A *P*-value less than 0.05 was considered significant.

Results

Allergen-specific lymphocyte proliferative reactivity

Allergen-specific lymphoproliferation to the allergen of treatment was measured at the initiation of SLIT (phase I), after 2 weeks (dose escalating phase, phase II), and after 8 weeks of therapy (dose maintenance period, phase III). Throughout the study period, proliferative activity of PBMC in response to the SLIT allergen did not change significantly ($n = 13$). Alterations in control cultures (recombinant IL-2 and tetanus toxoid) did not indicate any trend either (Fig. 1).

Patterns of intracellular and secreted cytokines

Levels of IL-2, IL-4, IFN-γ, and IL-10 in CD4⁺ and CD8⁺ lymphocytes were analyzed in eight patients at the given

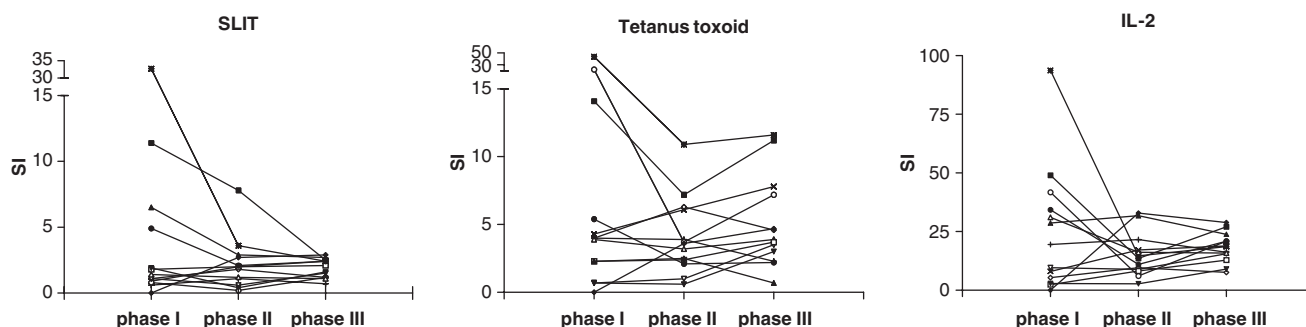


Fig. 1. Allergen-specific lymphoproliferation assays at initiation of sublingual immunotherapy (SLIT) (phase I), after 2 weeks of therapy (dose incremental period, phase II), and after 8 weeks of therapy (dose maintenance period, phase III). Peripheral blood mononuclear cells were stimulated with the allergen of treatment (Dermatophagoides pteronyssinus (Der p) 1, recombinant timothy grass pollen allergen 1 (rPhl p 1), recombinant birch pollen allergen 1 (rBet v 1), respectively), recombinant IL-2 and tetanus toxoid were used as controls. Lymphocyte proliferative activity was measured at day 6 by 3H-thymidine uptake, results are given as stimulation index (SI), $n = 13$.

Table 1. Intracellular cytokine production ($n = 8$) and levels of secreted cytokines ($n = 9$) at initiation of SLIT (phase I), after 2 weeks of therapy (dose incremental period, phase II), and after 8 weeks of therapy (dose maintenance period, phase III)

	SLIT			Tetanus toxoid			Control		
	Median	Range	<i>P</i>	Median	Range	<i>P</i>	Median	Range	<i>P</i>
Intracellular cytokines ($n = 8$)									
IL-4 ⁺ CD4 ⁺ (%)									
Phase I	3.91	0.51–7.44		3.13	1.15–8.95		2.97	1.04–10.32	
Phase II	3.24	0.24–14.67	0.779	2.28	0.59–8.33	0.401	2.13	1.02–6.00	0.327
Phase III	2.08	0.41–12.534	0.889	2.41	0.37–9.91	0.237	3.42	0.45–8.13	0.779
IFN- γ ⁺ CD4 ⁺ (%)									
Phase I	2.51	1.66–7.73		2.68	1.54–7.77		2.3	1.46–7.15	
Phase II	3.47	0.82–8.39	0.779	2.47	0.82–4.58	0.401	2.97	1.57–5.37	0.352
Phase III	2.44	1.47–5.78	0.889	1.68	0.86–3.35	0.128	1.88	0.84–6.31	0.018*
IFN- γ ⁺ CD8 ⁺ (%)									
Phase I	17.01	6.63–35.88		15.25	10.23–40.71		15.42	12.14–47.48	
Phase II	17.33	5.67–31.12	0.208	16.11	6.5–29.37	0.674	15.95	11.12–24.06	0.237
Phase III	13.44	7.27–38.08	0.575	14.16	6.61–30.79	0.237	11.8	6.87–31.31	0.236
Secreted cytokines ($n = 9$)									
IL-4 (pg/mL)									
Phase I	37.11	4.04–105.97		33.32	4.93–642.63		17.96	2.47–333.401	
phase II	21.09	0.523–412	0.767	14.75	6.65–418.68	0.11	22.51	2.77–259.29	0.594
Phase III	17.38	3.89–159.78	0.674	23.19	1.94–77.04	0.11	15.32	1.64–377.51	0.594
IFN- γ (pg/mL)									
Phase I	814.8	78.4–2741.7		814.8	147.12–6845.94		536.8	157.6–2960.04	
Phase II	737.23	85.7–4202.7	1	897.54	170.36–4686.12	0.441	406.6	61.22–2458.72	0.859
Phase III	352.5	142.4–4023.66	0.515	482.89	108.46–1121.04	0.123	394	133–8748.72	0.515
IL-10 (pg/mL)									
Phase I	3.44	0.83–14.89		3.09	1.9–67.04		3.2	0.59–30.56	
Phase II	5.68	0.36–82.78	0.612	4.69	0.48–160.6	0.441	4.27	0.48–65.75	0.139
Phase III	6.84	1.19–26.9	0.161	4.51	0.95–15.2	0.407	6.12	0.83–65.41	1
TGF- β_1 (pg/mL)									
Phase I	84.93	0–185.71		125.5	0–311.0		115.11	0–208.04	
Phase II	90.76	0–201.79	0.886	129.75	0–233.93	0.674	146.25	0–262.84	0.327
Phase III	95.17	0–264.29	0.208	92.19	0–277.68	0.208	60.42	0–219.64	0.31

PBMC were co-cultured with the allergen of treatment (Der p 1, rPhl p 1, or rBet v 1) for 12 days. Tetanus toxoid and the cell suspension without allergen served as controls. Levels of intracellular cytokines were measured by flowcytometry, IL-4⁺ and IFN- γ ⁺ cells are given as percentages of CD4⁺ and CD8⁺ lymphocytes. Patterns of secreted cytokines (IL-4, IFN- γ , IL-10, and TGF- β_1) were measured in culture-supernatants by ELISA.

* $P < 0.05$, Wilcoxon matched-pairs signed-rank test.

SLIT, sublingual immunotherapy; PBMC, Peripheral blood mononuclear cells; Der p 1, Dermatophagoides pteronyssinus; rPhl p 1, recombinant timothy grass pollen allergen 1; rBet v 1, recombinant birch pollen allergen 1.

times after 12 days of culture. There were no significant variations in IL-4⁺CD4⁺, IFN- γ ⁺CD8⁺, IL-2⁺CD4⁺, and IL-2⁺CD8⁺ cells in allergen-stimulated and control cultures, respectively (Table 1, data for IL-2 not shown). A significant decrease in IFN- γ ⁺CD4⁺ cells was seen from phases I to III in the negative control ($P = 0.018$). As percentages of IL-4⁺CD8⁺, IL-10⁺CD4⁺, and IL-10⁺CD8⁺ cells were very low ($< 0.5\%$, 5% in almost all experiments, data not shown) and could not be clearly discriminated from the negative populations, these data were excluded from statistical analysis.

Levels of IL-4, IFN- γ , IL-10, and TGF- β_1 secreted by allergen-specific PBMC after 12 days of culture were measured in nine patients and did not change signifi-

cantly throughout the study period. The same applies to cytokine levels measured in control cultures (Table 1).

Immunoglobulin levels

Levels of IgE, IgG1, IgG4, and IgA specific for recombinant Der p 1, Der p 2, Der p 5, and Der p 7 and complete HDM extract were detected in plasma samples of seven patients receiving SLIT against HDM. Neither significant alterations nor consistent trends in allergen-specific IgE, IgG1 or IgA during the studied first 8 weeks of SLIT were observed (Fig. 2). No allergen-specific IgG4 could be detected (data not shown).

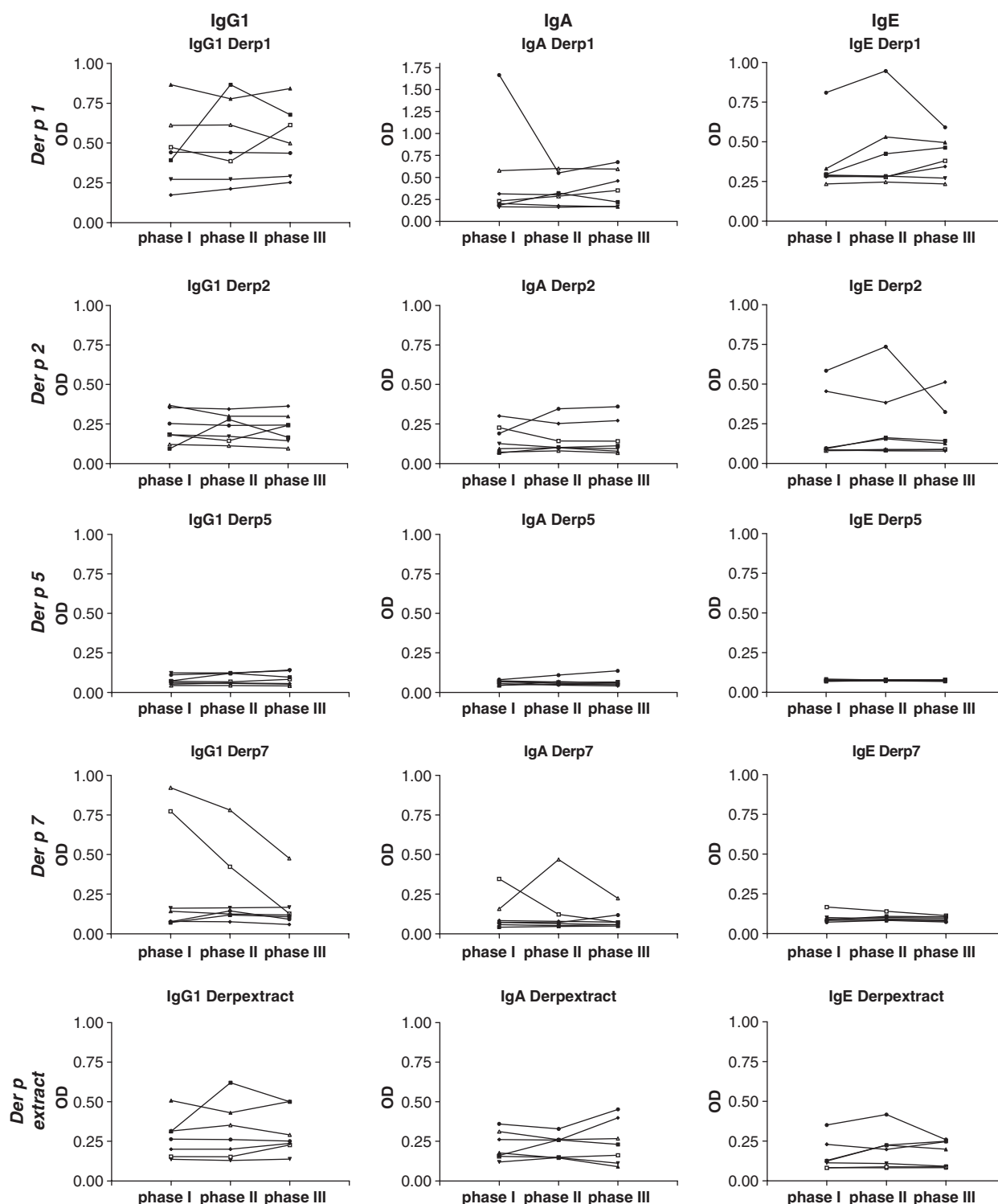


Fig. 2. IgG1, IgA, and IgE antibodies specific for recombinant *Dermatophagoides pteronyssinus* (Der p) 1, Der p 2, Der p 5, Der p 7, and Der p extract. Plasma samples from seven patients receiving sublingual immunotherapy (SLIT) against house dust mite were obtained at initiation of SLIT (phase I), after 2 weeks of therapy (dose incremental period, phase II), and after 8 weeks of therapy (dose maintenance period, phase III). Results are given in OD.

Discussion

In order to identify early systemic immunologic changes during effective SLIT in children, we monitored allergen-

specific proliferation, and intracellular as well as secreted cytokines in PBMC, according to data on the immunologic mechanisms of subcutaneous-specific immunotherapy [32–38]. Analysing allergen-specific proliferative activity

of PBMC ($n=13$), we did not observe any significant changes in stimulation indices of allergen-stimulated cultures or controls (Fig. 1). These results are concordant with the so far single placebo-controlled study on cellular immunologic mechanisms of SLIT involving children, finding no significant differences in allergen-specific lymphocyte proliferation between groups after 1 and 2 years of SLIT against grass pollen. However, no longitudinal evaluation within groups was performed in this study [39].

Regarding SLIT-induced alterations in intracellular ($n=8$) as well as secreted allergen-specific cytokine patterns ($n=9$), we observed no effect on the T-helper type 2 (Th2)-associated cytokine IL-4 or the Th1-dedicated cytokine IFN- γ , which concurs with results from other studies [14, 39]. In addition, neither IL-10 nor TGF- β_1 , two cytokines associated with the induction of immunological tolerance, which are currently discussed as key factors in subcutaneous immunotherapy [33, 38, 40], were affected during the first 8 weeks of SLIT (Table 1). To enhance the validity of our cytokine measurements, we decided to use two methods of detection – flow cytometry and ELISA – as values of intracellular and secreted cytokines have been reported not to match exactly, especially in atopic individuals [41]. In our study both sources of data were consistent for IL-4 and IFN- γ , but we observed a slight discrepancy in levels of intracellular and secreted IL-10. While the percentages of IL-10⁺CD4⁺ and IL-10⁺CD8⁺ lymphocytes were below 0.5%, 5% in almost every patient and were hence excluded from statistical analysis (data not shown), secreted cytokine could be measured, even though at low levels (Table 1). Most likely, other cells than lymphocytes represented the source of IL-10 in supernatants. The significant drop in IFN- γ levels in CD4⁺ cells from phases I to III in control cultures ($P=0.018$) is difficult to discuss (Table 1). It can probably be considered as an artefact, as it was neither reflected in CD8⁺IFN- γ ⁺ cells, nor in levels of secreted cytokine.

In contrast to data on cellular mechanisms, a higher number of studies has evaluated the alteration of allergen-specific Ig levels during SLIT. However, some authors observed alterations in allergen-specific IgE and/or IgG4 [11–18], whereas others did not find any changes [19–22, 39]. After 8 weeks of SLIT, we did not find significant alterations in specific IgE and IgG1 levels to HDM extract in plasma samples of seven children. These results were further confirmed by detection of specific antibodies to the recombinant HDM allergens Der p 1, Der p 2, Der p 5, and Der p 7 (Fig. 2). Specific IgG4 was not induced throughout the study period. In addition, we measured allergen-specific IgA because TGF- β_1 favours IgA-isotype switching [42], but neither TGF- β_1 nor IgA were affected by SLIT (Table 1 and Fig. 2). However, it has to be considered that the study period (first 8 weeks of therapy) might have been too short to achieve statistically significant alterations in Ig levels, although in some other

studies specific IgA was not altered after 1 year [22] and specific IgE and IgG4 levels did not change even after 2 years of therapy [20, 39].

Certainly, peripheral blood may not be suitable for investigating effects of local immunotherapy and following salivary or other secretory IgA might be more reasonable. Recently, the blunting of the seasonal peak in nasal-specific IgE but not in serum IgE in children suffering from allergic rhinoconjunctivitis to grass pollen in the course of high-dose SLIT was observed, suggesting local immunologic effects in the absence of systemic effects [43]. Further evidence for a pivotal role of the local immune system in the immunologic mechanisms of SLIT has been provided by a study showing no direct absorption of sublingually administered radio-labelled *Parietaria judaica* through the oral mucosa but a fraction of the extract could be detected at the sublingual level for up to 20 h. This led to the hypothesis of antigen up-take and -processing by local dendritic cells, which after migration to the local lymph nodes act as antigen-presenting cells and may contribute to the induction of tolerance by modifying T cell responses [44].

On the other hand, as the organs intended to be treated by SLIT are the nose and the lung, and the immunologic effects leading to improvement of symptoms have to be transferred to these organs, it would be reasonable to expect systemic immunologic changes.

With SLIT, a safe and easily applicable way of specific immunotherapy has been found. Yet the underlying immunologic mechanism is still unknown. In this study, we did not observe any early systemic immunologic changes in the first 8 weeks of clinically successful SLIT in paediatric patients with allergic rhinoconjunctivitis and/or asthma. As our results are concordant with other studies, we assume that the immunologic mechanisms of SLIT may differ from those of SIT, and local immunologic effects potentially occur without alterations of systemic parameters. We conclude that further studies investigating parameters of the systemic and in particular the local immune system of the target organs are required.

Acknowledgement

The authors thank Allergopharma, Reinbeck, Germany for providing the affinity purified HDM allergen 1 (Der p 1). This work was supported by the Austrian Science Fund (FWF/SFB), F 1817-B13, F 1807-B04, and F1803.

References

- 1 Bousquet J, Lockey R, Malling H-J, eds. WHO Position Paper. Allergen immunotherapy: therapeutic vaccines for allergic diseases. *Allergy* 1998; 53 (Suppl.):1–42.
- 2 Lockey R, Benedict L, Turkeltaub P, Bukantz S. Fatalities from immunotherapy and skin testing. *J Allergy Clin Immunol* 1987; 79:660–77.

- 3 Committee on the Safety of Medicines. Desensitizing vaccines. *BMJ* 1986; 293:948.
- 4 Bousquet J, Van Cauwenberge P, eds. Allergic rhinitis and its impact on asthma (ARIA). *J Allergy Clin Immunol* 2001; 108 (Suppl.):S147–334.
- 5 Holt P, McMenamin C. Defence against allergic sensitization in the healthy lung: the role of inhalation tolerance. *Clin Exp Allergy* 1989; 19:255–62.
- 6 Van Wilsem E, Van Hoogstraten I, Brevé J, Scheper R, Kraal G. Dendritic cells of the oral mucosa and the induction of oral tolerance. A local affair. *Immunology* 1994; 83:128–32.
- 7 Zemann B, Schwaerzler C, Griot-Wenk M *et al.* Oral administration of specific antigens to allergy-prone infant dogs induces IL-10 and TGF-beta expression and prevents allergy in adult life. *J Allergy Clin Immunol* 2003; 111:1069–75.
- 8 Wilson D, Torres LM, Durham S. Sublingual immunotherapy for allergic rhinitis (Cochrane Review). In: *The Cochrane Library*. Chichester: Wiley, 2003.
- 9 Reider N. Sublingual immunotherapy for allergic rhinoconjunctivitis – the seeming and the real. *Int Arch Allergy Immunol* 2005; 137:181–6.
- 10 Sopo S, Macchiaiolo M, Zorzi G, Tripodi S. Sublingual immunotherapy in asthma and rhinoconjunctivitis; systematic review of paediatric literature. *Arch Dis Child* 2004; 89:620–4.
- 11 Bufe A, Ziegler-Kirbach E, Stoeckmann E *et al.* Efficacy of sublingual swallow immunotherapy in children with severe grass pollen allergic symptoms: a double-blind placebo-controlled study. *Allergy* 2004; 59:498–504.
- 12 Bousquet J, Scheinmann P, Guinépain M *et al.* Sublingual-swallow immunotherapy (SLIT) in patients with asthma due to house-dust mites: a double-blind, placebo-controlled study. *Allergy* 1999; 54:249–60.
- 13 Clavel R, Bousquet J, André C. Clinical efficacy of sublingual-swallow immunotherapy: a double-blind, placebo-controlled trial of a standardized five-grass-pollen extract in rhinitis. *Allergy* 1998; 53:493–8.
- 14 Fanta C, Bohle B, Hirt W *et al.* Systemic immunological changes induced by administration of grass pollen allergens via the oral mucosa during sublingual immunotherapy. *Int Arch Allergy Immunol* 1999; 120:218–24.
- 15 Hirsch T, Sähn M, Leupold W. Double-blind placebo-controlled study of sublingual immunotherapy with house dust mite extract (D.pt.) in children. *Pediatr Allergy Immunol* 1997; 8:21–7.
- 16 La Rosa M, Ranno C, André C, Lorina C, Osca MA, Canonica GW. Double-blind placebo-controlled evaluation of sublingual-swallow immunotherapy with standardized *Parietaria judaica* extract in children with allergic rhinoconjunctivitis. *J Allergy Clin Immunol* 1999; 104:425–32.
- 17 Pradalier A, Basset D, Claudel A *et al.* Sublingual-swallow immunotherapy (SLIT) with a standardized five-grass-pollen extract (drops and sublingual tablets) versus placebo in seasonal rhinitis. *Allergy* 1999; 54:819–28.
- 18 Torres Lima M, Wilson D, Pitkin L *et al.* Grass pollen sublingual immunotherapy for seasonal rhinoconjunctivitis: a randomized controlled trial. *Clin Exp Allergy* 2002; 32:507–14.
- 19 Nelson H, Oppenheimer J, Vatsia G, Buchmeier A. A double-blind, placebo-controlled evaluation of sublingual immunotherapy with standardized cat extract. *J Allergy Clin Immunol* 1993; 92:229–36.
- 20 Pajno GB, Morabito L, Barberio G, Parmiani S. Clinical and immunologic effects of long-term sublingual immunotherapy in asthmatic children sensitized to mites: a double-blind, placebo-controlled study. *Allergy* 2000; 55:842–9.
- 21 Purello-D'Ambrosio F, Gangemi S, Isola S *et al.* Sublingual immunotherapy: a double-blind, placebo-controlled trial with *Parietaria judaica* extract standardized in mass units in patients with rhinoconjunctivitis, asthma, or both. *Allergy* 1999; 54: 968–73.
- 22 Bahceciler N, Arıkan C, Taylor A *et al.* Impact of sublingual immunotherapy on specific antibody levels in asthmatic children allergic to house dust mites. *Int Arch Allergy Immunol* 2005; 136:287–94.
- 23 Passalacqua G, Albano M, Riccio A *et al.* Clinical and immunologic effects of a rush sublingual immunotherapy to *parietaria* species: a double-blind, placebo-controlled trial. *J Allergy Clin Immunol* 1999; 104:964–8.
- 24 Passalacqua G, Albano M, Fregonese L *et al.* Randomised controlled trial of local allergoid immunotherapy on allergic inflammation in mite-induced rhinoconjunctivitis. *Lancet* 1998; 351:629–32.
- 25 Kopp M, Zehle C, Pichler J *et al.* Allergen-specific T cell reactivity in cord blood: the influence of maternal cytokine production. *Clin Exp Allergy* 2001; 31:1536–43.
- 26 Kopp M, Pichler J, Halmerbauer G *et al.* Culture conditions for the detection of allergen-specific t-cell reactivity in cord blood: influence of cell number. *Pediatr Allergy Immunol* 2000; 11:4–11.
- 27 Szépfalusi Z, Pichler J, Elsässer S *et al.* Transplacental priming of the human immune system with environmental allergens can occur early in gestation. *J Allergy Clin Immunol* 2000; 106: 530–6.
- 28 Pichler J, Gerstmayr M, Szépfalusi Z, Urbanek R, Peterlik M, Willheim M. 1- α ,25(OH) $_2$ D $_3$ inhibits not only Th1 but also Th2 differentiation in human cord blood T cells. *Pediatr Res* 2002; 52:12–8.
- 29 Eiwegger T, Stahl B, Schmitt J *et al.* Human milk-derived oligosaccharides and plant-derived oligosaccharides stimulate cytokine production of cord blood T-cells *in vitro*. *Pediatr Res* 2004; 56:536–40.
- 30 Bohle B, Breitwieser A, Zwölfer B *et al.* A novel approach to specific allergy treatment: the recombinant fusion protein of a bacterial cell surface (S-layer) protein and the major birch pollen allergen Bet v 1 (rSbsC-Bet v 1) combines reduced allergenicity with immunomodulating capacity. *J Immunol* 2004; 172: 6642–8.
- 31 Mothes N, Heinzkill M, Drachenberg K *et al.* Allergen-specific immunotherapy with a monophosphoryl lipid A-adjuvant vaccine: reduced seasonally boosted immunoglobulin E production and inhibition of basophil histamine release by therapy-induced blocking antibodies. *Clin Exp Allergy* 2003; 33:1198–208.
- 32 Ebner C, Siemann U, Bohle B *et al.* Immunological changes during specific immunotherapy of grass pollen allergy: reduced lymphoproliferative responses to allergen and shift from Th $_2$ to Th $_1$ in T-cell clones specific for phlp1, a major grass pollen allergen. *Clin Exp Allergy* 1997; 27:1007–15.
- 33 Jutel M, Akdis M, Budak F *et al.* IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal

- immunity and specific immunotherapy. *Eur J Immunol* 2003; 33:1205–14.
- 34 Majori M, Caminati A, Corradi M, Brianti E, Scarpa S, Pesci A. T-cell cytokine pattern at three time points during specific immunotherapy for mite-sensitive asthma. *Clin Exp Allergy* 2000; 30:341–7.
 - 35 Secrist H, Chelen C, Wen Y, Marshall J, Umetsu D. Allergen Immunotherapy decreases interleukin 4 production in CD4+ T cells from allergic individuals. *J Exp Med* 1993; 178:2123–30.
 - 36 Benjaponpitak S, Oro A, Maguire P, Marinkovich V, DeKruyff R, Umetsu D. The kinetics of change in cytokine production by CD4+ T cells during conventional allergen immunotherapy. *J Allergy Clin Immunol* 1999; 103:468–75.
 - 37 Akdis C, Akdis M, Blesken T *et al.* Epitope specific T cell tolerance to phospholipase A2 in bee venom immunotherapy and recovery by IL-2 and IL-15 *in vitro*. *J Clin Invest* 1996; 98:1676–83.
 - 38 Akdis C, Blesken T, Akdis M, Wüthrich B, Blaser K. Role of IL10 in specific immunotherapy. *J Clin Invest* 1998; 102:98–106.
 - 39 Rolinck-Werninghaus C, Kopp M, Liebke C, Lange J, Wahn U, Niggemann B. Lack of Detectable Alterations in Immune Responses during Sublingual Immunotherapy in Children with Seasonal Allergic Rhinoconjunctivitis to Grass Pollen. *Int Arch Allergy Immunol* 2005; 136:134–41.
 - 40 Gardner L, Thien F, Douglass J, Rolland JM, O'Hehir R. Induction of T 'regulatory' cells by standardized house dust mite immunotherapy: an increase in CD4+ CD25+ interleukin-10+ T cells expressing peripheral tissue trafficking markers. *Clin Exp Allergy* 2004; 34:1209–19.
 - 41 Tang M, Kemp A. Production and secretion of interferon-gamma (IFN-gamma) in children with atopic dermatitis. *Clin Exp Immunol* 1994; 95:66–72.
 - 42 Kim P, Eckmann L, Lee W, Han W, Kagnoff M. Cholera toxin and cholera toxin B subunit induce IgA switching through the action of TGF-beta 1. *J Immunol* 1998; 160:1198–203.
 - 43 Marcucci F, Sensi L, Di Cara G, Incorvaia C, Frati F. Dose dependence of immunological response to sublingual immunotherapy. *Allergy* 2005; 60:952–6.
 - 44 Bagnasco M, Mariani G, Passalacqua G *et al.* Absorption and distribution kinetics of the major *Parietaria judaica* allergen (par j1) administered by noninjectable routes in healthy human beings. *J Allergy Clin Immunol* 1997; 100: 122–9.