

Systemic Immunological Changes Induced by Administration of Grass Pollen Allergens via the Oral Mucosa during Sublingual Immunotherapy

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Key Words

Type I allergy · IgE · Sublingual immunotherapy ·
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Abstract

Twenty-four patients suffering from grass pollen allergy underwent sublingual immunotherapy (SLIT) with standardized grass pollen extract for 1 year. In order to investigate immunological changes induced by the administration of allergens via the oral mucosa, the SLIT-spit method was applied. The cumulative dose of approximately 80 µg of major allergen (grass group 5 allergen), was relatively low. During the time of treatment, we could observe a significant increase in the levels of specific IgG and IgG4 antibodies. However, the titers of allergen-specific IgE antibodies showed a significant increase in the course of SLIT as well. Analyzing lymphoproliferative responses, a significant decrease in reactivity in response to stimulation with complete grass pollen extract ($p = 0.001$) and to recombinant Phl p 1 (a major allergen of timothy grass, $p < 0.001$) could be observed, indicating the induction of immunological tolerance. Proliferative responses to a control antigen (tetanus toxoid) were not influenced by the treatment.

The first two authors contributed equally to the work.

At different time points during SLIT, allergen (Phl p 1)-specific T cell clones (TCC) were established from the peripheral blood of the patients. Cytokine production by allergen-stimulated T cells did not reveal any changes consistent with immune deviation, i.e. the ratio of Th1/Th2 TCC did not change during SLIT. In conclusion, we provide evidence that sublingual treatment leads to systemic changes in immunoreactivity to the administered allergen.

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Introduction

Hyposensitization by subcutaneous injection of increasing doses of allergen extracts leads to significant improvement of allergy symptoms. Numerous controlled clinical studies have demonstrated the efficacy of specific immunotherapy in patients suffering from type I allergy [1–3]. However, the treatment consists of many injections and the occurrence of side effects (typical allergic symptoms) is possible [4–6]. Therefore, immunotherapy methods combining effective hyposensitization with improved acceptance and safety for the patient have been investigated for many years. In experimental animals it has been demonstrated that the administration of antigens via mucosal surfaces leads to a state of tolerance rather than to immune re-

sponses. In this respect, mucosal treatment with antigens is presently under investigation as a novel therapy for certain autoimmune diseases [7, 8]. Accordingly, one strategy in specific immunotherapy of type I allergy was to change the conventional (parenteral) route of administration and to apply the allergen extract via a mucosal surface, e.g. sublingually or nasally.

In recent years, several double-blind, placebo-controlled studies have demonstrated that sublingual immunotherapy (SLIT) was clinically effective in pollen-induced [9–11] and in house-dust-mite-induced rhinitis and asthma [12–14]. These studies provided evidence that SLIT could represent a viable alternative to subcutaneous immunotherapy [3]. The immunological mechanisms operative in conventional, injection-based specific immunotherapy have been investigated recently [15–18]. Specific immunotherapy led to a significant reduction in lymphoproliferative reactivity in response to the injected allergen and to a modulation of the typical Th2-biased allergic immune response. Moreover, specific immunotherapy induced a marked increase in allergen-specific IgG, which seemed, however, not to be associated with the clinical outcome of the treatment [19]. So far, one published study investigated the immunological changes during mucosal immunotherapy [20]. Giannarini et al. [20] demonstrated that local, nasal immunotherapy induced a decrease in allergen-specific T cell responses. As a consequence, these authors described reduced cytokine production in the absence of a clearcut Th2-Th1 shift. In the present study, we examined immunological changes induced by SLIT. We analyzed changes in allergen-specific antibodies, lymphoproliferative responses and changes in Th subpopulations during 1 year of treatment with standardized grass pollen extracts.

Materials and Methods

Patients

24 patients (8 male, 16 female, aged 14–47 years; mean 25.5 years) volunteered to participate in the study after informed consent. They were selected according to the following criteria: clinical symptoms (rhinitis and/or seasonal bronchial asthma) during the grass pollen season, positive skin prick test to grass pollen extracts and specific IgE to grass pollen (more than class 3) as determined by RAST-CAP. None of the patients had previously received conventional immunotherapy.

Sublingual Immunotherapy

SLIT was performed using a standardized allergen extract containing 6 species of grass pollens (ALK-oral SQ200, ALK-Abello, Horsholm, Denmark); the concentrations of the solutions were as follows: 0.05, 0.5, 5, 50 and 500 STU/ml (STU = specific treatment units), respectively. A schedule of increasing doses was used, with

drops to be taken by the sublingual route once daily, increasing from 1 to 10 drops for each vial until the maximal dose (10 drops of 500 STU/ml) was reached. This dose contains approximately 0.625 µg major allergen (according to the producer, 500 STU/ml corresponds to 1.25 µg/ml of the group 5 major grass pollen allergens). The treatment was started in autumn and was followed by a maintenance therapy with 10 drops of 500 STU/ml three times a week for 1 year, i.e. a total dose of approximately 80 µg of group 5 major allergen per patient. As we wanted to observe immunological changes induced by the sublingual administration exclusively, patients were instructed not to swallow, but to retain the extract for 2 min under the tongue and then to discard the solution.

Study Design

Blood samples were taken and evaluations were performed at three time points: before SLIT (autumn; phase I), after the dose increase phase (December/January; phase II) and after 1 year of treatment with the maintenance dose (autumn; phase III). IgE immunoblot analysis using complete grass pollen extract was performed in order to observe qualitative changes in the IgE-binding pattern in the course of treatment. To investigate quantitative changes in the antibody levels, rPhl p 1-specific IgE, IgG and IgG4 were measured by an established ELISA system. Cellular tests included lymphoproliferative responses of peripheral blood mononuclear cells (PBMC) to grass pollen extract and rPhl p 1, the establishment of rPhl p 1-specific T cell clones (TCC) with characterization of their cytokine profiles.

Allergens, Antigens for Experimental Work

Pollen from *Phleum pratense* (timothy grass) was obtained from Allergon (Engelholm, Sweden). Complete grass pollen extract was produced as described [21]. Recombinant Phl p 1 [22], a highly purified nonfusion protein was obtained from Biomay, Linz, Austria. Lyophilized tetanus toxoid was obtained from Calbiochem, San Diego, Calif., USA. For use in cell culture, lyophilized proteins were dissolved in tissue culture medium (Bio Whittaker, Walkersville, Md., USA) and filtrated under sterile conditions [21].

Antibody Determinations

ELISA: Microtiter plates (M129A, Dynatech MT, Zug, Switzerland) were coated with rPhl p 1 (2 µg/ml in PBS); thereafter ELISA was performed as described [18] using mouse-anti-human IgG, IgG4 and IgE antibodies (Pharmingen, San Diego, Calif., USA) and sheep-anti-mouse Ig-POX-labeled antibodies (Amersham, UK) for detection. Sera were titrated starting from an 1:20 dilution to 1:1280 for IgG and IgG4, and 1:2 to 1:32 for IgE determination. Immunoblots with complete extract of *P. pratense* were performed as described [18]. In order to guarantee comparable results, all sera were kept frozen until the end of the study and antibody determinations were performed in one experiment.

Proliferation Assays

10^5 PBMC were cultured in duplicates in 96-well plates (200 µl) in serum-free Ultra Culture Medium (Bio Whittaker) supplemented with 2 mM/l glutamine and 2×10^{-5} M 2-ME, in the presence of rPhl p 1 and complete grass pollen extract for 6 days at 37°C, 5% CO₂, humidified atmosphere. In the last 16 h of culture, [³H]-thymidine (0.5 µCi per well) was added and the incorporated radioactivity was measured by scintillation counting [18]. Allergens were titrated in the concentration range: 1.5, 3, 6, 12, 25, 50 µg/ml. The concentration inducing the best proliferative response before the start of the treatment was in-

dividually determined and used for evaluating the course of the T cell reactivity during the study period. Tetanus toxoid was used in triplicates at the concentrations of 1, 0.25, 0.05 µg/ml in proliferation assays. For control experiments with tetanus toxoid, 11 subjects were selected. These individuals had been vaccinated against *Clostridium tetani* with tetanus toxoid within the last 5 years but did not receive booster injections during the study period.

Allergen-Specific T Cell Lines

Allergen-specific short-term T lymphocyte lines (TCL) were obtained by a previously described technique [23]. Briefly, 0.5×10^6 PBMC were stimulated with individual optimal concentrations (20–40 µg/ml) of purified rPhl p 1 in serum-free medium (Bio Whittaker) supplemented with 2 mM/l glutamine and 2×10^{-5} M 2-ME in 24-well flat-bottomed culture plates (3524 Costar, Cambridge, Mass., USA). After 5 days of culturing, human rIL-2 (10 U/ml, Boehringer, Mannheim, Germany) was added, and the culture was continued for additional 7 days. In case proliferation could be observed, allergen-specific TCC were established by limiting dilution.

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 (Nunc, Denmark) in the presence of 2×10^5 irradiated (50 Gy) allogeneic PBMC as 'feeder cells', 0.5% PHA v/v (Gibco, Grand Island, Mich., USA) and rIL-2 (4 U/well) in culture medium. Growing microcultures were expanded at weekly intervals with fresh allogeneic irradiated feeder cells and rIL-2. The specificity of TCC was assessed in proliferation assays as soon as the cell number reached 2×10^5 .

5×10^4 T cell blasts were incubated in duplicate cultures in 96-well plates in the presence of 10^5 autologous PBMC as antigen-presenting cells (APC) and rPhl p 1 for 48 h at 37°C, 5% CO₂ in humidified atmosphere. After pulsing for 16 h with 0.5 µCi per well of [³H]-thymidine, cells were harvested and the incorporated radioactivity was measured by scintillation counting. When the stimulation index (SI; ratio of cpm obtained in cultures with TCC plus autologous APC plus antigen, to cpm obtained in cultures containing TCC and APC alone) was greater than 10, responses were considered as positive. Phl p 1-specific TCC were thereafter expanded by alternating turns of stimulation with autologous irradiated APC and Phl p 1, or with allogeneic feeder cells and rIL-2.

Analysis of the Phenotype of TCC

The phenotype of TCC was analyzed by means of flow cytometry, using a fluorescence-activated cell sorter (FACScan; Becton Dickinson, Mountain View, Calif., USA). For analysis, the FITC-labeled monoclonal antibodies anti-Leu 4/CD3, anti-Leu 3a/CD4, anti-Leu 2a/CD8, anti-TCR αβ WT 31 and anti-TCR γδ I (Becton Dickinson) were used as described [23].

Stimulation for Cytokine Production

10^6 TCC were washed and incubated with 10^6 irradiated (50 Gy) autologous APC in the presence of rPhl p 1 (5 µg/ml) in 1 ml medium. Supernatants from allergen-stimulated cultures were collected after 24 h of incubation. Cytokine levels in the supernatants were measured using ELISA systems purchased from Quantakine R&D systems, Minneapolis, Minn., USA (IL-4, sensitivity threshold: 3 pg/ml), Medgenix Diagnostics, Fleurus, The Netherlands (IFN-γ, sensitivity threshold: 1.5 pg/ml). Cultures containing TCC and APC alone were used as negative controls [23].

Results

Antibody Determinations

The mean values of allergen-specific IgG and IgG4 antibodies showed a significant increase after 1 year of therapy (IgG: $p = 0.01$, IgG4: $p = 0.003$ paired-sample t test; fig. 1). Moreover, a significant increase in rPhl p 1-specific IgE serum levels was found in SLIT-treated patients after 1 year of treatment ($p = 0.001$, paired-sample t test; fig. 1). This picture was reflected by IgE immunoblots, where we could observe an increased IgE-binding to grass pollen allergens in the course of the treatment (fig. 2).

Proliferation Assays and T Cell Cloning Efficiency

We observed a significant decrease in the lymphoproliferative reactivity (stimulation indices) in response to grass pollen extract ($p = 0.001$, Mann-Whitney U test) and to rPhl p 1 ($p < 0.001$) after the dose increase phase (phase II), this effect remained highly significant after 1 year of SLIT (phase III; $p < 0.0001$) (fig. 3). Using the absolute values of proliferation (Δcpm) instead of relative values (SI) led to identical results, i.e. a significant decrease in allergen-induced proliferation. Proliferative responses of PBMC in response to stimulation with tetanus toxoid were generally low, and did not change significantly in the same blood samples during the course of grass pollen SLIT. On three occasions (before: I, after the phase of dose-increase: II, after 1 year: III) during SLIT treatment, Phl p 1-specific TCC were produced.

Analysis of Phenotype of TCC

Fifty-nine TCC could be expanded in vitro to cell numbers which allowed the completion of the above-mentioned experimental setup. All TCC exhibited the Th phenotype (CD3+, CD4+, CD8-) and expressed the αβ-TCR. TCC were classified into the Th subsets by calculating the IL-4:IFN-γ ratio. Before SLIT, 27 TCC derived from 10 different individuals could be analyzed. Fifteen (55.6%) of these TCC had the Th2 type of cytokine production, whereas only 2 (7.4 %) of the TCC were Th1. 10 TCC (37%) produced both: IL-4 and IFN-γ, or neither of these cytokines (in spite of a strong proliferative response) and were classified as 'Th0'. After the phase of dose increase, 8/15 (53.3%) TCC were Th2, 3/15 (20%) Th0, and 4/15 (26%) Th1. After 1 year of treatment, the ratio of Th2:Th0:Th1 was 52.9:35.2:11.7. In total, during the course of SLIT, no marked differences between the distributions T-helper subsets could be observed. Characteristics of Phl p 1-specific TCC isolated during the course of SLIT are summarized in table 1.

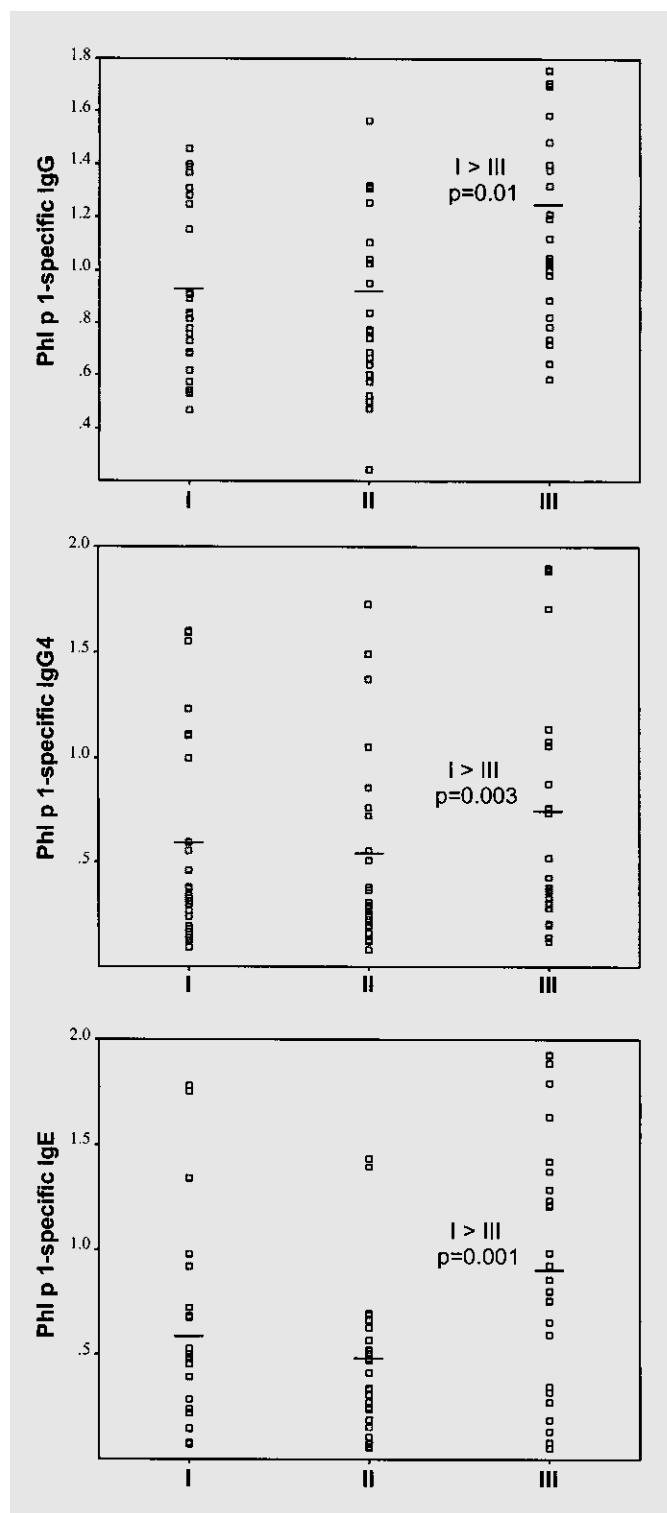


Fig. 1. Changes in serum Phl p 1-specific IgG, IgG4 and IgE during SLIT. I: Before SLIT; II: after the dose increase phase; III: after 1 year of SLIT. The difference between antibody levels at identical time points in the year (autumn, before therapy, phase I) and after 1 year of SLIT (autumn, after 1 year treatment, phase III) was statistically significant (paired-sample t test).

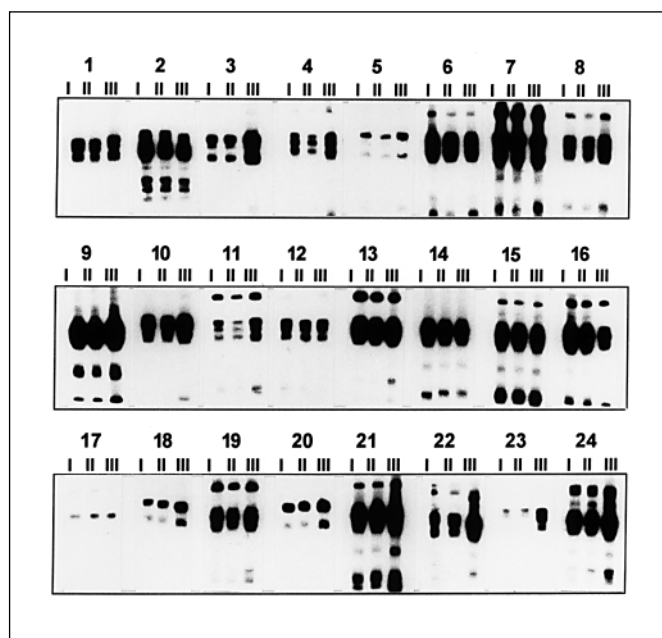


Fig. 2. IgE immunoblot: serum-IgE reactivity to allergens in complete timothy grass pollen extract during SLIT. I: Before SLIT; II: after the dose increase phase; III: after 1 year of SLIT.

Discussion

Noninjective routes of immunotherapy are thought to be valuable therapeutic options for type I allergy [3]. The main objective of using the local, mucosal route of vaccine administration is to provide a well-tolerated, effective treatment. Secondly, improved patient compliance can be expected, because no injections are given and the treatment can be carried out at home. In placebo-controlled double-blind studies, SLIT has been demonstrated to be clinically effective [9–14]. In this study, we analyzed systemic immunological changes associated with the sublingual administration of low doses of allergen extracts for 1 year. Peripheral blood was collected on three occasions and the course of specific antibodies and lymphoproliferative responses to the administered grass pollen extract and to a recombinant grass pollen major allergen: Phl p 1 [21, 22] were assessed. Previously published results demonstrated a marked increase in allergen-specific IgG and IgG4 antibodies in patients treated with conventional subcutaneous immunotherapy, whereas no significant changes in the levels of allergen-specific IgE could be observed [18, 19]. RPhl p 1-ELISA with sera of SLIT-treated patients also revealed a significant quantitative increase in specific IgG and IgG4 antibodies during the treatment (fig. 1). However, we also

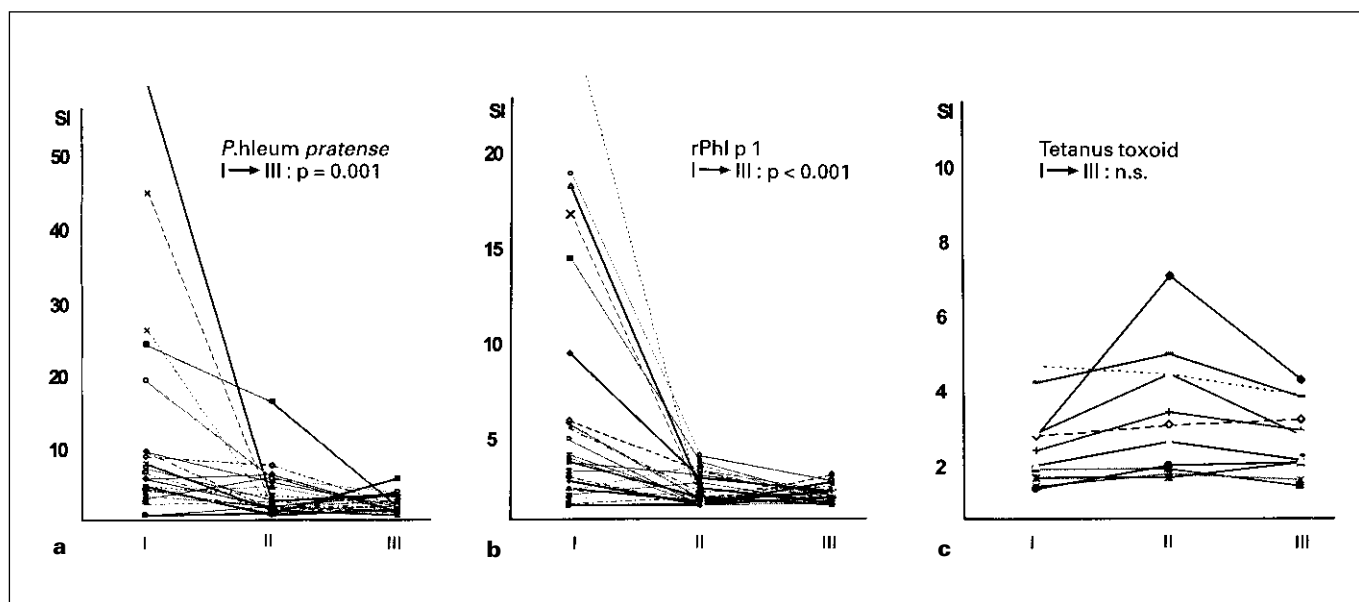


Fig. 3. Proliferation assays using PBMC incubated with complete timothy pollen extract (**a**), recombinant Phl p 1 (**b**), and tetanus toxoid (**c**), respectively. The allergen/antigen concentration individually inducing the best proliferation before SLIT (I) was used throughout the study. II: After the dose increase phase; III: after 1 year of SLIT.

detected a significant ($p = 0.01$, fig. 1) increase in allergen-specific IgE antibodies. This trend was also visible in IgE immunoblot experiments with complete timothy grass pollen extracts (fig. 2). Interestingly, similar observations were reported in high-dose SLIT-swallow therapy where a significant increase of all isotypes including house-dust-mite-specific IgE could be observed [14]. Simultaneously to this increase in antibody levels, we measured a marked decrease in polyclonal lymphoproliferative reactivity in response to stimulation with timothy grass pollen extract and to rPhl p 1 (fig. 3). The discrepant finding that the antibody titers rose while T cell reactivity decreased, is difficult to interpret. It underlines, however, that successful allergen immunotherapy is associated with changes at the T cell level rather than with alterations in quantity or distribution of antibodies. Using our cell culture system we could not find evidence for increased production of cytokines associated with the induction of immunological tolerance, in particular TGF- β and IL-10 [7, 8, 24–27] (not shown). However, T lymphocytes cloned from peripheral blood are certainly not a suitable test material for this purpose.

During conventional immunotherapy (i.e. injection immunotherapy), a shift from a Th2 to a more Th1/Th0 type immune response to the injected allergen can be observed [15–18]. Analyzing the cytokine production of Phl p 1-specific TCC established during 1 year of SLIT (table 1), we could not detect any significant change in the patterns of IL-

4 or IFN- γ , the markers for the Th2 and Th1 type, respectively.

In conclusion, we describe systemic immunological effects induced by the administration of allergens via the oral mucosa. We hypothesize that local APCs (probably mucosal dendritic cells) take up the allergen and migrate to the regional lymphoid tissue. It is known that mucosal-associated lymphoid tissue has a special aptitude to induce immune tolerance [7, 8]. On the other hand, previous studies provided no evidence for resorption of allergens through the sublingual mucosa [28]. In accordance with the results published by Giannarini et al. [20], analyzing the events occurring during local nasal immunotherapy, we conclude that sublingual administration of allergen led to a significant decrease in T cell reactivity (tolerance) in the absence of a clearcut Th2-Th1 shift (immunodeviation). The allergen doses applied in this study were low [11, 12] compared to other published papers [9, 14, 29, 30]. It is possible that immunodeviation can be achieved by applying higher doses and/or by extending the treatment period.

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Table 1. IL-4 and IFN- γ production of Phl p 1-specific T cell clones established during SLIT

TCC I	IL-4 pg/ml	IFN- γ pg/ml	Th subset ^a	TCC II	IL-4 pg/ml	IFN- γ pg/ml	Th subset ^a	TCC III	IL-4 pg/ml	IFN- γ pg/ml	Th subset ^a
BE 34-1	2,079.2	<1.5	2	BE 41-2	2,006	50	2	BE 18-3	2,006	<1.5	2
BE 65-1	2,034	<1.5	2								
BI 6-1	<3	<1.5	0	BI 25-2	1,886	51	2	BI 22-3	1,700	<1.5	2
BI 4-1	<3	<1.5	0								
BI 22-1	2,936	<1.5	2								
BI 43-1	32	37	0								
CH 5-1	1,966	<1.5	2	CH 8-2	530	<1.5	2	CH 93-3	15	<1.5	2
CH 18-1	1,864	10	2	CH 19-2	1,946	<1.5	2	CH 49-3	14	30	0
CH 52-1	200.8	230	0	CH 47-2	1,832	<1.5	2				
				CH 11-2	4.4	<1.5	0				
CL 31-1	6.6	<1.5	0	CL 8-2	10	<1.5	0	CL 26-3	186	<1.5	2
CL 62-1	4	<1.5	0	CL 12-2	126	<1.5	2	CL 47-3	<3	<1.5	0
CL 65-1	11	<1.5	0	CL 31-2	15.2	3,200	1	CL 49-3	16.8	<1.5	2
								CL 50-3	4	<1.5	0
CS 17-1	144.5	191	0					CS 7-3	118	<1.5	2
CS 37-1	21.5	3,200	1					CS 21-3	1,002	4	2
CS 40-1	225.5	<1.5	2								
JO 27-1	1,609	135	2	JO 4-2	<3	3,300	1	JO 19-3	10.8	10	0
				JO 90-2	<3	30	1				
MI 10-1	1,917	0.7	2	MI 15-2	<3	3,250	1	MI 2-3	<3	3,590	1
MI 35-1	<3	3,570	1					MI 115-3	15.8	1,790	1
PA 3-1	341	<1.5	2								
PA 37-1	<3	<1.5	0								
PA 46-1	<3	<1.5	0								
PA 21-1	111.6	10	2								
PA 24-1	160	<1.5	2								
TI 4-1	2,023	190	2	TI 18-2	<3	<1.5	0	TI 28-3	60	<1.5	2
TU 12-1	594	22.5	2	TU 2-2	2122	<1.5	2	TU 54-3	2,102	<1.5	2
TU 18-1	2106	<1.5	2	TU 37-2	2144	10	2	TU 70-3	14.4	<1.5	0
TU 25-1	2052	4.4	2					TU 95-3	<3	<1.5	0

TCC I, TCC II, TCC III = produced before (TCC I), after the dose increase phase (TCC II) and after 1 year of SLIT (TCC III).

^a 10⁶ clone cells were stimulated in the presence of 10⁶ irradiated PBMC and optimal concentration of rPhl p 1. IL-4 and IFN- γ were measured in culture supernatants by specific ELISA. TCC with IL-4:IFN- γ >10 were classified Th2, IFN- γ :IL-4>10 Th1, all others TH0.

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