

Modified T-cell activation pattern during specific immunotherapy (SIT) in cat-allergic patients

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Summary

Objective The aim of the study was to analyse early effects of specific immunotherapy (SIT) on immune functions in cat-allergic patients.

Methods Immunological responses of peripheral blood mononuclear cells from eight cat-allergic patients were analysed before and after SIT in comparison with 11 nonallergic controls. Cells were stimulated *in vitro* with either bacterial superantigen, mitogen, or cat allergen. Production of IL-12 and TH1/TH2 cytokines was analysed by ELISA and lymphocyte subset distribution was assessed by flow-cytometry.

Results We found a significantly reduced secretion of IL-12 ($P < 0.05$) from cells of allergic individuals compared with the controls. This finding was associated with significantly lower IFN- γ production after stimulation with allergen ($P < 0.05$) that did not increase during SIT. However, no significant differences were seen after stimulation with mitogen indicating an allergen specific IFN- γ secretion response in allergic individuals. Prior to SIT IL-5 production was significantly higher in cells of allergic donors stimulated with allergen < 0.005 or mitogen (< 0.05). After reaching the maintenance dose for SIT, allergen-induced IL-5 production returned to normal levels, whereas it remained elevated after stimulation with mitogen. These changes were associated with a reduced frequency of CD45 RO T cells following SIT.

Conclusion These results suggest that SIT exerts early effects on allergen-specific T-cell responses with selective inhibition of the up-regulated TH2 immune response.

Keywords: cat allergy, naive and memory cells, specific immunotherapy, TH1/TH2 cytokines

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Introduction

Specific immunotherapy (SIT) with purified and standardized allergen extracts is indispensable in the treatment of immediate-type hypersensitivity responses of the upper and lower airways [1]. Although clinical efficacy of this type of therapy has been demonstrated in several double-blind placebo-controlled studies, the underlying mechanisms of action are still not fully understood [2–4]. There are

indications that one mode of action of SIT is a shift in the allergen-specific T-cell response patterns [5]. Development of allergic rhinitis and bronchial asthma due to hypersensitivity response against aero-allergens is associated with increased allergen-specific IgE production which is under control of IL-4 (and IL-13) producing activated CD4 and CD8 T cells [6,7]. Eosinophilia in these patients depends in part on elevated IL-5 production by the same T-cell subset [8,9]. SIT alters this type of T-cell response as illustrated by decreased production of type 2 cytokines, enhanced production of IFN- γ and inhibition of the T-cell-dependent cutaneous late phase responses [10,11]. Independent modes of action may cause these effects on T-cell responses.

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Possibilities include a shift of TH2 to TH1-like effector cells, new stimulation of TH1 cells and down-regulation of allergen-specific T-cell functions by induction of tolerance or anergy. The development and function of allergen-specific T-cell responses, however, is under control of allergen-presenting cells that shape the type and nature of effector T cells via delivery of cognate and non-cognate signals. Little is known about the effect of SIT on monocytes and macrophages and whether the function of this cell type is modified by SIT.

The present study was designed to prospectively follow a clinically well-defined group of patients with allergic respiratory symptoms due to cat allergy through treatment with conventional allergen extract using standard SIT protocol. Conventional SIT consists of two phases. In an initial first phase the maximum tolerated dosage is reached incrementally, with injections administered over a prolonged maintenance period. To monitor the clinical effect of SIT, patients were examined before SIT and after reaching the maintenance dosage generally after 3 months of treatment. The clinical parameters included titrated skin tests and bronchial provocation with histamine/methacholine and allergen. These parameters were compared with changes of lymphocyte and monocyte functions.

Prior to SIT, cat-allergic patients were characterized by an elevated production of the pro-inflammatory cytokine IL-5 and reduced amounts of IFN- γ following *in vitro* allergen stimulation. This imbalance was associated with reduced secretion of IL-12 by monocytes, a cytokine known to induce type 1 T-cell responses. Following the induction phase of SIT, a profound re-organization of allergen-specific T-cell functions was noted with marked inhibition of IL-5 production, although no alteration in IFN- γ and IL-12 secretion could be observed. These changes were associated with a reduced frequency of (primed) CD45 RO T cells following SIT and increase in (naive) CD45 RA T cells. Thus, the result might reflect a marked anti-allergic and anti-inflammatory effect of SIT that is allergen specific and operates at the T-cell/antigen-presenting cell (APC) level.

Materials and methods

Study population

The study group consisted of eight adult atopic patients (mean age 45 years) sensitized to cat allergen (Fel d) and 15 nonatopic control persons. All patients had at least a 3-year history of rhinoconjunctivitis and allergic asthma requiring drug treatment. Sensitization to cat allergen was confirmed by a positive skin prick test, specific serum IgE (measured by Pharmacia CAP FEIA, Uppsala, Sweden) and a specific bronchial provocation test to cat allergen. Control persons had neither history nor clinical signs of allergic disease, and

testing for total and specific serum IgE was negative. All patients gave informed consent. Continued therapy for inhaled corticosteroids, sodium chromoglycate, nedocromile, or theophylline were not allowed for at least 4 weeks before the first visit and during the entire study period. Cats were not allowed in the household or as part in the routine environmental exposure. In addition, no subjects had seasonal allergies. The treatment for asthma consisted of inhaled β_2 -agonists only. This study was approved by the local ethics committee.

Study design

All patients attended two prestudy visits which included a prick test and a bronchial provocation titration test. Heparinized blood was drawn for further studies on lymphocyte functions as explained below. SIT with cat allergen (Fel d) was performed weekly with increasing dosages of cat allergen for 12–14 weeks. For SIT, cat allergen extract was obtained from ALK, Copenhagen, Denmark. Subcutaneous injections were given according to the manufacturer's dosage and interval instructions. During the monitored time course the individual maximum treatment dosage was established ranging from 10 000 to 410 000 SQ. Treatment was continued with this dosage. After reaching the individual maximum allergen dosage, skin prick test, bronchial provocation and lymphocyte function tests were repeated. Skin prick tests, bronchial provocation tests and measurements of allergen-specific IgE values were repeated again after 1.5 years. Missing values in certain readouts are indicated in figure legends.

Titration skin prick tests

Commercial preparations of cat allergen extract were used for skin prick testing (ALK). Titration of allergen was performed by diluting the extract in 0.9% NaCl to final concentrations of 1000, 3300, 10 000, 33 000, 100 000, 333 000, and 600 000 SQ/mL which is equivalent to $\approx 90 \mu\text{g}$ of Fel d 1/mL. Tests were carried out in duplicate. The area of the resulting weal after 15 min was determined using an AMS Optomax V Image Analyser (Respiratory Care Centre, Hämevilla, Finland).

Titration bronchial provocations

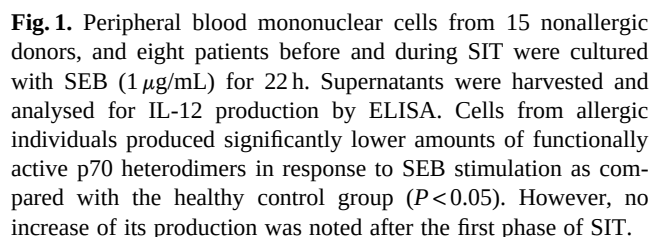
Bronchial challenge was performed with a Spira Elector 2 Inhalation dosimeter (Respiratory Care Centre). Aerosolization time at each dose level was set to 0.5 s, which delivered $7.1 \mu\text{L}$ per breath. FEV₁ was measured before, eight times following inhalation of the saline diluent as negative control and after inhalation of the allergen solutions. Nebulization of the allergen was performed in

Heparinized blood (60 mL) was drawn from the patients prior to and after reaching the maximum individual allergen dosage of SIT, as well as from the healthy control donors. PBMCs were obtained by gradient centrifugation using Lymphoprep and cultures were carried out as previously described [12]. 2×10^6 cells/mL were cultured in RPMI 1640 cell culture medium (Serva, Germany) supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL), 100 U/mL penicillin, 100 μ g/mL streptomycin. For the analysis of IL-4, IL-5 and IFN- γ production cells were either stimulated with PHA (5 μ g/mL) as a mitogen (Sigma Chemicals, Munich, Germany) or Fel d (50 μ g/mL) as the specific allergen (ALK). Production of IL-12 was induced by stimulation with the superantigen SEB (1 μ g/mL, Seva, Germany). Cells were cultured at 37°C in a 95% air, 5% CO₂ atmosphere. As shown before [12] and confirmed in pilot experiments, reproducible results were obtained after a culture period of 24 h. Supernatants were harvested and stored at -20°C until further analysis.

Supernatants of cell cultures were analysed for cytokine production by the sandwich ELISA technique. Antibodies for capture and detection of IL-4 and IL-5 as well as recombinant human IL-4 and IL-5 as standards were purchased from PharMingen, San Diego CA, USA. For detection of IFN- γ , capture and detection antibodies as well as recombinant human IFN- γ as a calibrator were purchased from Medgenix Diagnostics. ELISAs were performed according to the manufacturer's protocol. For detection of the functional active heterodimer p70 of IL-12 a commercially available ELISA was used (R & D Systems, Biermann Diagnostika, Bad Nauheim, Germany). All detection antibodies were biotinylated and development was performed with streptavidine peroxidase (Calbiochem, Bad Soden, Germany) followed by the substrate tetramethylbenzidine/TMB (Fluka, Neu-Ulm, Germany).

Identification of lymphocyte subpopulations as well as their differentiation and activation status was carried out by flow-cytometry. PBMCs of freshly drawn blood were stained

IL-12 is considered to be an important cytokine involved in the determination of the differentiation of T-cell subpopulations towards a TH1 phenotype. It was not detectable in unstimulated and allergen-stimulated cell cultures from control and allergic individuals. Therefore cells were stimulated with SEB (1 $\mu\text{g/mL}$), which is known to induce APCs to produce measurable amounts of the functionally active p70 heterodimers of IL-12 *in vitro*. As shown in Fig. 1, cells of allergic donors produced significantly lower amounts of



IL-12 in response to stimulation with SEB as compared with the healthy control group ($P < 0.05$). However, no increase in production could be seen on reaching the individual maximum allergen dosage during SIT after 3 months of treatment. These results indicate differences in IL-12 production between allergic patients and control individuals and further suggest that there is still an IL-12 suppressive action after the beginning of SIT.

The production of IFN- γ following stimulation with the cat allergen extract is significantly lower in the allergic donor group both prior to (<0.005) and after (<0.05) beginning SIT when compared with the control group (Fig. 2b). This result is in line with the IL-12 measurements, showing that by the time of reaching maximum treatment dosage no measurable switch of T cells towards a TH1 cytokine profile had occurred. Stimulation of the same cells with the T-cell mitogen PHA, however, resulted in normal production of IFN- γ in both groups (pre-SIT: maximum dosage 210 pg/mL; median 195 pg/mL) which was not significantly different ($P > 0.05$) between allergic and healthy individuals (median: 249 pg/mL). This indicates an allergen-specific IFN- γ secretion response of allergic individuals, and depending on the type of stimulus, cells from allergic individuals can be induced to produce normal amounts of IFN- γ .

Marked effects were seen on IL-5 production. As shown in Fig. 2(a), prior to SIT, cells from allergic individuals produced significantly higher amounts of IL-5 when stimulated with allergen extract than the nonallergic control cells ($P < 0.01$). After 3 months of SIT, no significant differences in IL-5 production were detected between the two groups due to decreased IL-5 production in the SIT study groups. However, when cells were stimulated with the mitogen PHA, IL-5 production was high in cells from allergic individuals (median: 253 pg/mL) when compared with the

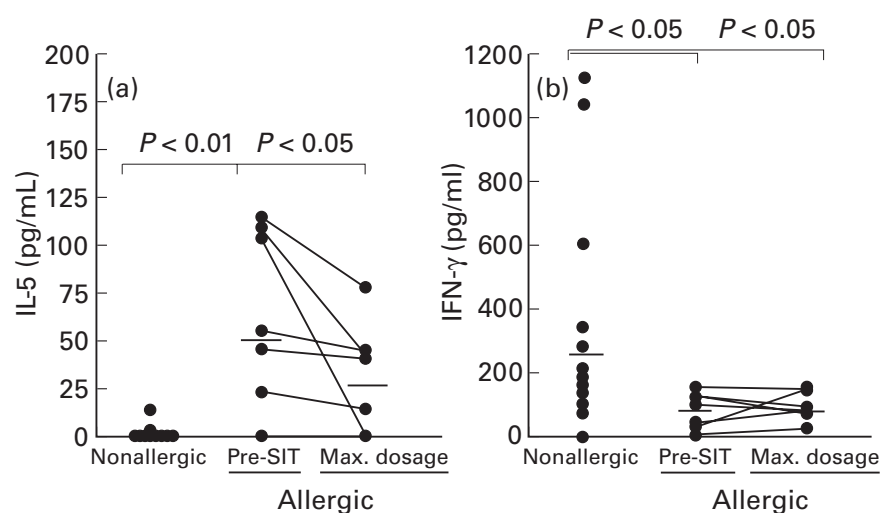
controls (median: 185 pg/mL) ($P < 0.05$) and remained on this level during SIT (median 288 pg/mL), indicating a selective effect of SIT on allergen-dependent immune responses at this stage of therapy.

When IL-4 production was analysed in this culture system, only very low amounts could be detected in supernatants after stimulation with allergen extract or PHA, and the values did not significantly differ between the allergic and nonallergic individuals. However, intracellular cytokine staining of PHA-stimulated cells revealed high amounts of IL-4-producing T cells in samples from the allergic donor group (data not shown). It remains unclear whether IL-4 was not secreted or was bound immediately after secretion.

In addition to the functional analysis, freshly isolated PBMCs from the donors were differentiated for their distribution of lymphocyte subpopulation by flow cytometry. During SIT, allergic individuals showed no significant differences in the distribution of CD3, CD4, CD8, CD16/CD56, HLADR, and CD19 expressing cells (data not shown). However, a marked change could be noted in the expression pattern of the CD45 isotypes RO vs RA during SIT. The expression of these different isotypes is supposed to distinguish cells from naive T cells (CD45 RA) and already primed ones (CD45 RO). Both CD4 (Fig. 3a) and CD8 T cells (Fig. 3c) from the allergic individuals showed a significantly higher percentage of CD45 RA T cells ($P < 0.05$) following SIT when compared with pre-treatment analysis. Conversely, CD45 RO T cells showed the opposite pattern (Fig. 3b,d).

Before and after reaching the maintenance dosage for SIT, patients were tested for their allergen reactivity by titrated skin prick test and bronchial provocation, and specific serum IgE measurements were carried out in parallel. As shown in Fig. 4, after 3 months of SIT an improvement in allergen-specific skin reactivity was

Fig. 2. Analysis of IL-5 (a) and IFN- γ (b) production in cell culture supernatants from seven patients as in Fig. 1. PBML were prepared and stimulated with allergen extract as described in material and methods.



detected ($P=0.059$). However, the PD_{20} of FEV_1 as a marker for bronchial reactivity after inhalation of cat allergen had not significantly improved so far, although a tendency of improvement was noted in five patients. No significant changes could be seen in serum IgE levels. In contrast, after 1.5 years significant improvements were detected on bronchial provocation ($P<0.05$) and specific IgE antibody titres ($P<0.05$; data not shown).

Discussion

Specific immunotherapy with purified and standardized allergen extracts has been clinically proven to be useful in the treatment of allergic patients with respiratory symptoms [4,5,13–16]. Despite the progress in care management, it is not fully understood how immunotherapy works or how immunological changes may develop. This study was aimed to analyse mechanisms that operate early and during onset of SIT. Since clinical efficacy of SIT with the same type of allergen preparation [13–16] has been well documented in comparable patient groups, we decided on an open trial design with prospective follow-up instead of a double-blind placebo-controlled study. Data before and during SIT were compared with nonatopic individuals. Clinical efficacy was evaluated after 3 months and 1.5 years, and significant changes in bronchial sensitivity to cat allergen as well as in reduction of specific IgE titres were found at the second investigation (data not shown). Several studies suggest that the primary effect of SIT might be a redirection of the skewed T-cell immune responses from a TH2 towards a TH1 effector cell pattern [17]. As demonstrated by several published observations the allergen-specific and allergen-dependent T-cell response in atopic patients is characterized by enhanced production of IL-4 and IL-5, compared with low production of IFN- γ , a cytokine attributed to TH1

effector cell functions [6,7,9,10]. These results could be confirmed in our experiments, although we were not able to detect high levels of IL-4 in supernatants of cultures from our study groups. However, using intracellular cytokine staining technique, IL-4 accumulation in PBMCs from allergic patients was shown to be much higher than in cells from control individuals. This predominant TH2 cytokine profile could also be seen after stimulation with the mitogen PHA, although IFN- γ production was comparable with the control group. This was paralleled by low amounts of IL-12 production following *in vitro* stimulation of accessory cells with SEB.

IL-12 is a cytokine known to be an initiator for the induction of a TH1 immune response leading to the development of cytotoxicity and IFN- γ production and inhibition of IL-4- and IL-5-dependent immune functions [18–20]. SEB acts as a potent inducer of IL-12 production as has been previously shown in mouse and men. In this context SEB acts like a mitogen for APCs [21]. The exact mechanisms involved in the induction of IL-12 production are not clearly understood. However, it is known that its secretion can be suppressed by TH2 cytokines such as IL-4 and IL-10 [22–27]. Therefore it remains unclear whether the decreased IL-12 production is a cause or result of the predominant TH2 profile measured here prior to SIT. In a mouse model for allergic asthma it has been shown that administration of IL-12 to sensitized mice could abolish airway hyperresponsiveness and pulmonary eosinophilia. This phenomenon was associated with increased production of IFN- γ and decreases in IL-4 and IL-5 [27]. This experiment also illustrates the important role of IL-12 for the regulation of the allergic immune response.

Already after about 3 months of treatment we found a marked improvement in skin prick test reactivity, associated with a trend to improvement in FEV_1 values following

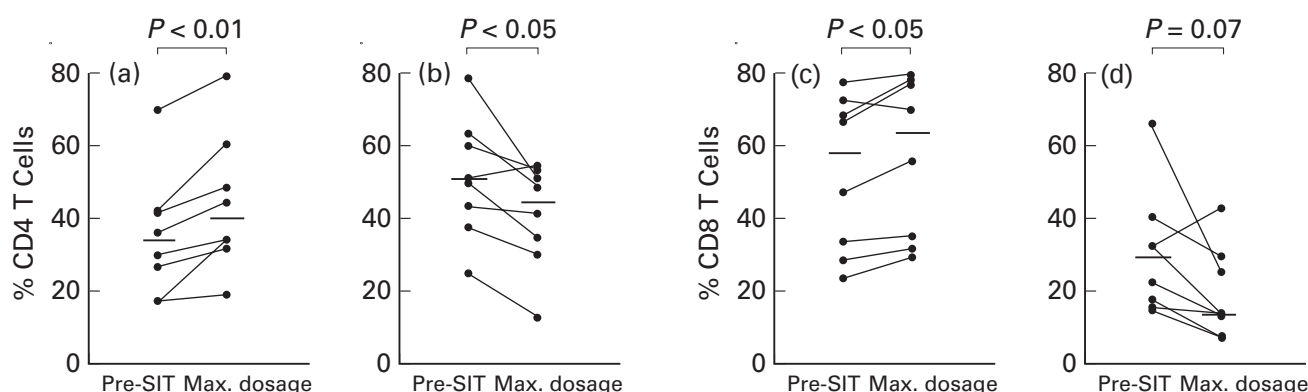


Fig. 3. Analysis of CD45 RA and CD45 RO expression. CD4 (a, b) and CD8 (c, d) T cells were double stained with antibodies directed against either the CD45 RA (a, c) or CD45 RO (b, d) isoform and the CD4 or CD8 molecule. Cells were analysed on a gate for lymphocytes. Staining was carried out as described in material and methods.

allergen provocation. However, there was no change in specific serum IgE antibody titres. The clinical effects were accompanied by certain changes in immune functions. In response to allergen stimulation, IL-5 production was significantly reduced and reached levels which did not significantly differ from those in cultures of healthy donors. IFN- γ production, however, was already reduced before SIT and remained low in response to allergen during SIT. In contrast, the immune response following mitogen (PHA) activation of the T cells showed an unchanged TH2 cell activation pattern with especially high IL-5 titres. Therefore, our results do not suggest the development of an SIT-induced T-cell shift from TH2 towards TH1 effector functions, but rather an improvement of allergic inflammation as a consequence of 'silencing' the allergen-specific immune response. Reduced IL-5 production might lead to a reduced recruitment and survival of eosinophils and a reduction of allergic inflammation [8,28–30]. It is still not clear whether an established TH2 cytokine secretion profile in a single T cell can be redirected to a TH1 profile. Recent work [31] has shown an IL-12 nonresponsiveness of allergen-specific TH2 clones, due to a total lack of IL-12-induced phosphorylation of STAT4. The authors postulate, that strongly polarized TH2 cells do not respond to IL-12, and therefore cannot be skewed to IFN- γ production. Other groups have demonstrated that the signal transducing β_2 subunit of the IL-12 receptor is selectively expressed on TH1 but not on TH2 cells and can be seen as an important step in the differentiation of the TH type [18,19,22,23,32–34].

One possibility to redirect the cytokine profile in response to a specific allergen would be the recruitment of naive unprimed T cells which are not committed to a specific cytokine profile. Cell surface marker staining of PBMCs from the allergic individuals prior to and during SIT in our

study revealed an increase in the CD45 RA vs CD45 RO cell subset within both the CD4 and CD8 T cell population. Since the CD45 RA isotype is supposed to define unprimed T cells, these results might reflect new recruitment of naive unprimed (CD45 RA⁺) CD4⁺ and CD8⁺ T cells into the peripheral lymphocyte pool. Alternatively, the decrease in CD45 RO cells may reflect recruitment of primed cells to the site of mucosal inflammation. Since this study was performed on cells from peripheral blood, further studies should be performed using tissue from local compartments. The shift in dose–response curves of titrated skin prick tests in all but one patient cannot be explained by a reduction in IgE production, since IgE titres did not significantly decrease during the initial phase of SIT. Therefore, we propose a different mechanism including an IgE-independent anergizing of mast cells at this early stage of SIT. The observed changes at least in terms of cytokine production were allergen dependent, since the response to mitogens remained unchanged.

This supports the concept of SIT as a specific anti-allergic therapy acting at least in part at the level of allergen-specific T cells. Induction of tolerance seems to be a desired effect of SIT. The normal immune response of healthy individuals to allergens is usually not characterized by a delayed-type hypersensitivity response with the development of TH1 IFN- γ secretion but rather by a non-responsiveness of T cells to the allergen. In fact several publications indicate the development of T-cell anergy following SIT including the demonstration of reduced proliferation in certain animal models [4,35,36]. Recently, several studies have demonstrated the development of a TH1 cell immune response in patients undergoing SIT as a consequence of an increase in IL-12 production [17]. These results are not necessarily in conflict with our data. One major difference is that all the other studies were carried out after a period of treatment of

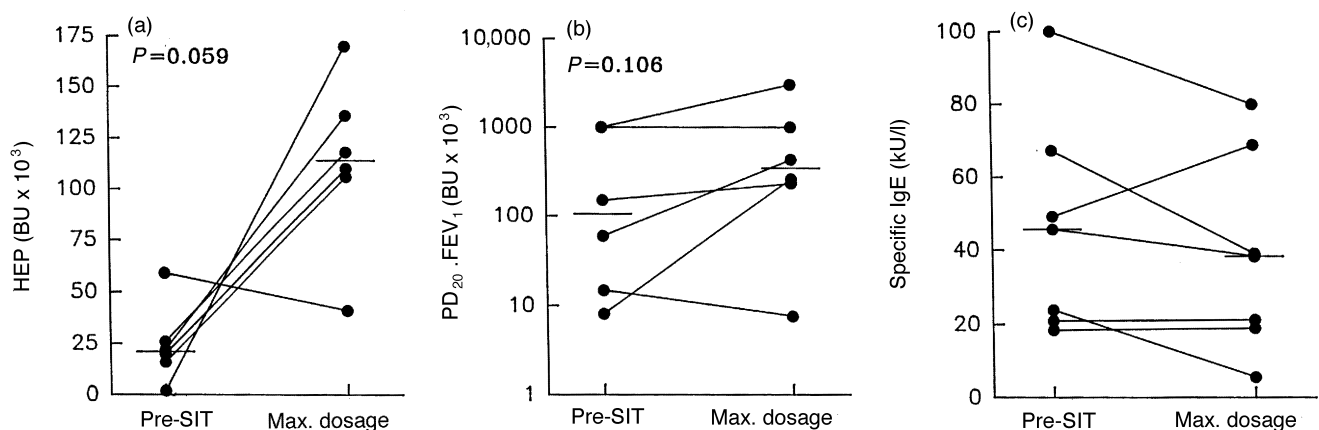


Fig. 4. Titrated skin prick tests ($n=6$), bronchial provocation tests ($n=6$) and measurements of allergen specific IgE ($n=7$) were performed before and during SIT as described in material and methods.

up to several years. If we take together all these observations it becomes likely that changes in specific immune functions take place in a dynamic fashion. We propose that the initial effect of SIT is a down-regulation of the allergen-specific type 2 T cells. The underlying molecular mechanism may be, for example, induction of anergy, development of tolerance or T-cell deletion. Prolonged treatment will then induce a type 1/type 0 T-cell response that represents a newly developed immune response on top of the eliminated type 2 effector population. To fully prove this concept extended studies are required which closely follow changes in specific T-cell functions in these patients at short intervals over time.

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This work is dedicated to the sixtieth birthday of Professor Eckart Kötting.

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