

Safety and Immunogenicity of a Cluster Specific Immunotherapy in Children with Bronchial Asthma and Mite Allergy

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Key Words

Specific immunotherapy • Cluster • Mite • Immunogenicity • Blocking antibodies • T cell subset transcription factors

Abstract

Background: Cluster specific immunotherapy (SIT) is a modern form of allergen immunotherapy allowing safe administration of high allergen doses in a short time interval compared to classic SIT. In the current study, we investigated the safety profile and immunological effect of cluster SIT in children with allergic asthma due to house dust mite allergy.

Methods: A total of 34 children (6–18 years) with allergic asthma were assigned to cluster (n = 22) or classic SIT (n = 12). To achieve a maintenance dose of allergen extract, cluster patients received 14 injections of house dust mite allergen within 6 weeks, whereas the classic SIT group received 14 injections within 14 weeks. Safety was monitored by recording adverse events. Immunogenicity was measured by specific IgG_{Mite} and IgG4_{Mite} by antibody-blocking properties on basophil activation, and by the T cell subset transcription factors Foxp3, T-bet, and GATA-3. **Results:** There were no significant differences in local and systemic side effects between the two groups. In the cluster group, serum levels of specific IgG_{Mite} (p < 0.001) and specific IgG4_{Mite} (p < 0.001) significantly increased after 8 weeks, while it took 12 weeks

in the classic SIT group. These data were confirmed by blocking CD63 expression as well as release of cysteinyl leukotrienes after in vitro basophil stimulation. No differences in transcription factor expression were found in the two groups. **Conclusion:** Cluster SIT is safe in children. Additionally, our data demonstrated an even more rapid induction of specific immune tolerance. Cluster SIT is an attractive alternative to conventional up-dosing schedules with fewer consultations for the patients.

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Introduction

To date, specific immunotherapy (SIT) is the only treatment modality for allergic disease, which has the potential to alter the natural course of the disease by the induction of immunotolerance [1, 2]. Clinical efficacy and safety of subcutaneous administration of standardized allergen extracts for the treatment of IgE-mediated allergic disorders have been well examined, as demonstrated in several randomized, double-blind controlled clinical trials [3–5]. Effectiveness of the treatment depends on the specific allergen the individual patient is

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Table 1. Patient baseline criteria

	Cluster (n = 20)	Classic (n = 10)
Age, years	10.0	8.5
FEV ₁ , % of predicted	104.6 (88.8–114.1)	108.9 (90.1–139.3)
eNO, ppb	44.7 (5.3–115.3)	32.2 (8.7–216.0)
Total IgE, kU/l	276.5	450.5
Specific IgE <i>Dermatophagoides pteronyssinus</i> , kU/l	105.5 (12.0–593.0)	95.8 (10.0–1,325)
Specific IgE <i>Dermatophagoides farinae</i> , kU/l	106.0 (6.8–331.0)	100.0 (6.5–616.0)
PD20 (cumulative dose), mg	150 (30–630)	150 (10–630)
Monosensitized (to mite), n	6	4
Polysensitized, n	12	6

sensitive to the quality and total amount of allergen applied, and the SIT schedule [6, 7].

However, the disadvantage of classic schedules is the time needed to reach the maintenance dose, accompanied by higher costs. To overcome these problems, several studies have been done in search of ideal schedules allowing the dose considered effective to be reached in the shortest possible time with the fewest adverse effects [8, 9]. Most of them demonstrated induction of tolerance without increase of systemic reactions in cluster schedules compared to the classic group [10–12].

In addition, recent studies found efficacy and changes in immunological parameters even more rapid in the cluster than in classic groups [8]. In this regard, a variety of immunological modifications have been reported after SIT, including changes in serum levels of specific IgE, IgG, and IgG4 antibodies [13, 14], blocking properties of specific antibodies to prevent mast cell/basophil activation/degranulation [15, 16], TH1 and TH2 cytokine secretion [17] and most recently the induction of allergen-specific regulatory T (Treg) cells [18, 19].

However, almost all studies enrolled predominately adults and data on clinical and immunological effects of cluster SIT in children are scarce. Thus, we evaluated the safety and immunogenicity of a cluster immunotherapy in children with house dust mite.

Material and Methods

Patients

Thirty-four 6- to 18-year-old patients were enrolled in the study (table 1). Inclusion criteria were mild to moderate asthma according to GINA guidelines [1], a positive skin prick test (Allergopharma Joachim Ganzer, Reinbek, Germany), and/or a specific immunoglobulin E to *Dermatophagoides pteronyssinus* (Der p 1), and a positive bronchial challenge to house dust mite with a consecutive decrease in forced expiratory volume in 1 s (FEV₁) of

at least 20%. Exclusion criteria were an FEV₁ of <70% of predicted value recorded on two or more occasions during the 2 weeks prior to randomization, a need for inhaled corticosteroids (>500 µg budesonide or beclomethasone/day or 250 µg fluticasone/day) for at least 3 months, and the use of systemic steroids or previous SIT with mite allergens. All parents and/or guardians provided written informed consent. The study was approved by the local institutional ethics committee and performed in accordance with the declarations of Helsinki (1964) and Edinburgh (2000).

Study Design

In an open randomized study we compared the tolerability and immunogenicity of a rapid regimen (cluster/group A) with the classic regimen (control/group B) in patients suffering from mite-allergic asthma (table 2).

Both groups were treated with a standardized aluminium hydroxide-absorbed house dust mite extract (Novo-Helisen® Depot Milbe, Der f 1 50%/Der p 1 50%; Allergopharma, Hamburg, Germany) and followed up for 16 weeks. The subjects were randomly allocated to a cluster (two thirds of the total group) or classic (one third) setting. In the classic setting, treatment was initiated at a dose of 5 therapeutic units (TU), increasing weekly until a dose of 5,000 TU was reached after 14 weeks. The initial cluster protocol started with three injections on the first treatment day, and was repeated in the 3 consecutive weeks (table 2). Since we observed an increase of systemic side effects (mainly coughing) in 5 patients in the cluster group after receiving a total dose of 1.2 ml (6,000 TU) at week 4, we changed the cluster regimen due to safety concerns as follows. At week 4, only two injections (a total of 0.6 ml = 3,000 TU) were given not exceeding the maintenance dose of 1 ml (5,000 TU) before week 6. Acute (within 30 min) and delayed reactions were graded according to the guidelines published by the European Academy of Allergology and Clinical Immunology [2], and the dose was adjusted accordingly. Exhaled NO (eNO) as well as blood samples for eosinophilic cationic protein (ECP), specific antibodies and blocking antibodies were taken at week 1, 8 and 16.

Clinical Procedures

The skin prick test was performed according to recommendations by using a panel of biological standardized allergens including Der p 1, Der f 1, cat and dog, mould mix, grass, and tree pollen mix (Allergopharma). The pulmonary function test was per-

formed with the MasterScreen® body plethysmograph (VIASYS Healthcare, Höchberg, Germany).

Bronchial provocation test was performed according to the method of Cockcroft et al. [20] with slight modifications as described [21]. Briefly, standardized native unmodified allergen extract (Allergopharma) was prepared according to the instruction of the manufacturer (containing sodium, phenol and water) resulting in aliquots of 5,000 mg/ml. The solution was delivered via a medic aid nebulizer and the aerosol provocation system APS® powered by compressed air (by VIASYS Healthcare). This system calculates the administered dose by breath from a constant flow and the inspiration time of any breath cycle, thus calculating the exact dose to be administered automatically. The dose of inhaled allergen was increased every 10 min according to the following pattern from step 1 to 7: 0, 10, 20, 40, 80, 160, 320 mg. Thus, the entire protocol delivers cumulative doses of 0, 10, 30, 70, 150, 310, 630 mg/ml. The challenge test was positive when the FEV₁ decrease was at least 20% from individual baseline or a cumulative dose of 630 mg had been achieved or significant clinical complaints were reported by the subjects (e.g., chest pain).

eNO was measured at a constant flow rate of 0.05 liter/s ($\pm 0\%$) during the time of the NO plateau generation using the NIOX® by Aerocrine (Solna, Sweden) according to the criteria of the American Thoracic Society. This chemiluminescence gas analyzer is sensitive to measure eNO at concentrations from 1.5 to 200 ppb with a deviation from mean value of ± 2.5 ppb < 50 ppb or $\pm 5\%$ of the measured value > 50 ppb.

Eosinophilic Cationic Protein

ECP was measured in serum using a two-sided chemiluminescent test (Immulate DPC, Bad Nauheim, Germany).

Specific Antibodies

Serum was collected at week 1, 8 and 16 and stored at -80°C until testing. Samples were analyzed for specific antibodies against Der p 1, IgE_{Mite} and IgG_{Mite}, using a two-sided chemiluminescent assay (Immulate DPC). Specific IgG₄_{Mite} was detected by ELISA technique (DST, Schwerin, Germany). Analysis was performed according to the manufacturer's instructions.

Blocking Antibodies

Cellular antigen stimulation test (CAST; Bühlmann Laboratories, Allschwil, Switzerland) was used to analyze inhibitory properties of serum antibodies. Therefore, leukocytes were isolated by dextran sedimentation from EDTA blood. Simultaneously, the patient's serum was incubated with dust mite extract (2 ng/ml) for 1 h at room temperature. A mixture of the patient's serum and dust mite extract was used for cellular stimulation for 40 min, supernatants were decanted and stored at -80°C . Ionomycin and anti-IgE antibody solutions were used as positive controls, patient's serum without dust mite extract as negative control. Cysteinyl leukotrienes (cysLT) were determined by ELISA technique according to the manufacturer's instructions.

In addition, cells from the pellet were triple labeled with anti-CD123-PE, anti-CD63-FITC, and anti-HLA-DR-Per-CP monoclonal antibodies (Becton Dickinson, San Jose, Calif., USA) and analyzed by flow cytometry (FACSCalibur, Becton Dickinson) equipped with an air-cooled 488-nm laser. On each sample, 300 basophils were collected and data analysis was performed with Cell Quest software (Becton Dickinson).

Table 2. Up-dosing schedules of cluster and classic SIT

Schedule weeks	Cluster SIT dose, ml	Classic SIT dose, ml
1	0.1 ^a , 0.2 ^a , 0.4 ^a	0.1 ^a
2	0.8 ^a , 0.1 ^b , 0.2 ^b	0.2 ^a
3	0.4 ^b , 0.8 ^b , 0.1 ^c	0.4 ^a
4	0.2 ^c , 0.4 ^c , (0.6 ^c) ^d	0.8 ^a
5	0.8 ^c	0.1 ^b
6	1.0 ^c	0.2 ^b
7	–	0.4 ^b
8	1.0 ^c	0.8 ^b
9	–	0.1 ^c
10	–	0.2 ^c
11	–	0.4 ^c
12	0.5 ^c	0.6 ^c
13	1.0 ^c	0.8 ^c
14	–	1.0 ^c
15	–	–
16	1.0 ^c	1.0 ^c
Cumulative dose	9.6 ml/33,825 TU 9.0 ml ^d /30,825 TU	7.1 ml/21,325 TU

^a Dose 50 TU. ^b Dose 500 TU. ^c Dose 5,000 TU. ^d Postamendment reduction of dose increasing in week 4 because of accumulation of side effects.

Purification of T Cells

Peripheral mononuclear blood lymphocytes from patients were obtained by centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). T cells were then enriched by magnetic cell sorting using a Pan T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Purifications were performed according to the manufacturer's instructions. Briefly, non-T cells (i.e., B cells, NK cells, dendritic cells, monocytes, granulocytes, and erythrocytes) were indirectly labeled magnetically by using a cocktail of biotin-conjugated antibodies against CD14, CD16, CD19, CD36, CD56, CD123, and glycophorin A, and anti-biotin microbeads. Isolation of highly pure T cells was achieved by depletion of magnetically labeled cells. Purity (85–90%) of the cells was tested by flow cytometry (Becton Dickinson).

T Cell Subset Transcription Factors

RNA was first purified using RNeasy Mini kit (Qiagen, Valencia, Calif., USA) and oligo-dT18-primed first-strand cDNAs were synthesized using OmniScript Reverse Transcriptase (Invitrogen, Carlsbad, Calif., USA). They were then used as a template for real-time polymerase chain reaction (PCR). PCRs were performed using specific primers for human forkhead box P3 (Foxp3): sense 5'-TCCCAAATCCAGTGCACCCAG-3', antisense 5'-TTCTCACATCCGGGCCACTTGC-3'; GATA-3: sense 5'-GGCATC-CAGACCAGAAACCG-3', antisense 5'-AAG GGC GAG ATG TGG CTC AG-3'; T-bet: sense 5'-GCTGTCAACCACTGGAAG-GAT-3', antisense 5'-TTGGTGTGGACTGAGATTGC-3', and the

Table 3. Clinical effects of cluster immunotherapy

	Cluster (n = 20) total/%	Classic (n = 10) total/%
Total local side effects	185/54.2	80/53.0
Redness	97/28.4	40/26.5
Swelling <5 cm	57/16.7	20/13.2
Swelling >5 cm	22/6.5	17/11.3
Painful swelling >3 h	8/2.3	3/2.0
Total systemic reactions	12/3.5	7/4.6
Cough	10/2.9	6/4.0
Dyspnea	2/0.6	1/0.7
Injections, n	341	151

house-keeping gene GAPDH: sense 5'-CGTCTTCACCAACAT-GGAGA-3', antisense 5'-CGGCCATCAGCCACAGTTT-3'.

Quantitative LightCycler PCR was performed by use of the QuantiTect® SYBR® Green PCR kit (Qiagen, Hilden, Germany). 12.5 µl QuantiTect SYBR Green Master Mix, 0.5 µl of each primer at a concentration of 50 pmol/µl and 10.5 µl water were added to 1 µl of cDNA, standard, or water (negative control). 20 µl of each mix were transferred into LightCycler capillaries (Roche, Mannheim, Germany) that were subjected to the following temperature profile within the LightCycler equipment (Roche): initial 15 min at 95°C to activate the enzyme, and 55 cycles of 95°C (15 s), 60°C (30 s), and 72°C (15 s). Finally, product homogeneity was verified by melting curve analysis. Calculation of crossing points was performed using the second derivative maximum method (included in LightCycler software) for the unknown samples and for DNA standards of known concentrations generated from purified PCR products of the respective gene. Unknown sample concentrations were then calculated from the standard curve. Different samples from each individual patient were adjusted to comparable GAPDH expression values and expression of the transcription factor genes was calculated relative to a respective standard.

Statistical Analysis

Data were analyzed using the statistical software SPSS 11.0 (SPSS, Chicago, Ill., USA) and presented as median and range. Within-group comparison was performed using a two-sided Wilcoxon matched-pair test, and differences between baseline and other time points were assessed by the Friedmann nonparametric repeated-measure test. Comparisons between groups were performed by using a Mann-Whitney U test. Values of $p < 0.05$ were considered statistically significant.

Results

Safety Profile

Thirty patients completed the study. Two girls and 2 boys dropped out (1 withdrawal of informed consent, 1 antibiotic treatment due to upper respiratory infection, 1

asthma exacerbation during grass pollen season, 1 non-compliance with treatment). The two groups were well matched for demography and clinical characteristics (table 1).

During the study, we performed 492 injections. The cluster group (n = 20) received 341 injections in comparison to the classic group (n = 10), who received 151 injections. Each subject in the cluster group received a dosage of 33,825 TU before and 30,825 TU after regimen modification in contrast to 21,325 TU in the classic group (table 2).

There were no significant differences in local and systemic side effects between the two groups (table 3). In the two groups, the most common local side effects were redness and swelling (<5 cm diameter). We observed local erythema in 28.4% of the cluster injections compared to 26.5% in the classic group. Swelling of more than 5 cm in diameter occurred in 6.5% of the injections in the cluster group in contrast to 11.3% in the classic group. Overall, we noticed 185 (54.2%) local side effects in the cluster group in comparison to 80 (53.0%) in the classic group (table 3). In general, these were mild and did not require treatment.

Systemic side effects were reported by less than 5% of subjects in each group (table 3). According to the grading scheme of the European Academy of Allergology and Clinical Immunology (EAACI) SIT guidelines, most systemic side effects were nonspecific or mild (grade 1–2). In 0.6% of cluster injections and in 0.7% of the classic group, we documented non-life-threatening grade 3 reactions (bronchial asthma). These subjects were allowed to continue the study at the discretion of the investigator if they wished to do so. No grade 4 (anaphylactic) reaction occurred. Overall, the cluster SIT caused systemic side effects in 12 (3.5%) in comparison to 7 (4.6%) in the classic SIT group.

Exhaled NO

There was a decrease of eNO in the cluster group, which failed to reach significance compared to the classic group (cluster week 1: 44.7 ppb, range: 5.30–115; week 2: 26.5 ppb, 7.60–90.4; week 3: 27.5 ppb, 8.90–95.1; classic week 1: 32.3 ppb, 8.70–216; week 2: 37.0 ppb, 6.60–190; week 3: 20.2 ppb, 7.70–95.0).

Eosinophilic Cationic Protein

There were no statistically significant differences in ECP between groups at baseline. During SIT we recorded a more rapid decline of ECP in the cluster group (week 1: 41.8 µg/l, 6.5–156; week 2: 22.5 µg/l, 10.5–92.6, $p < 0.05$;

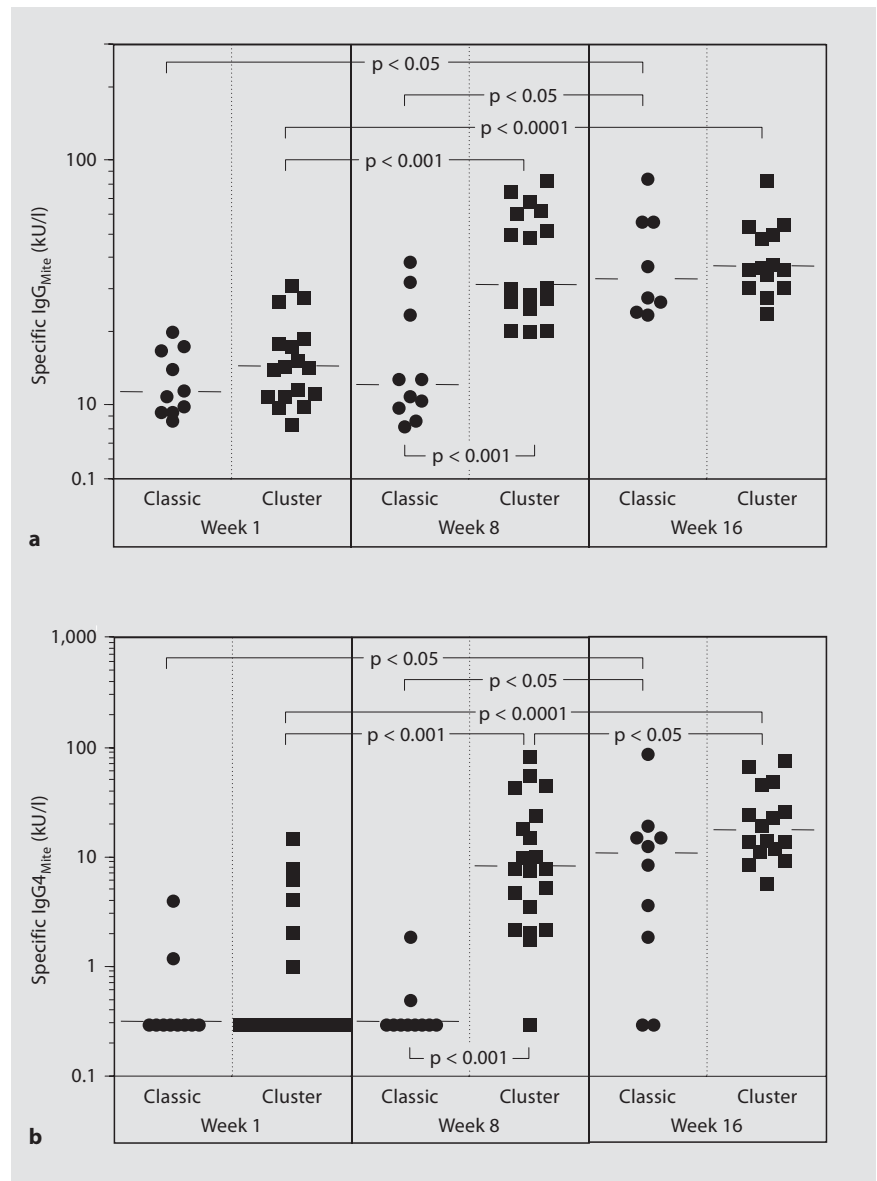


Fig. 1. Serum concentrations of specific IgG_{Mite} (Der p 1) (**a**) and specific IgG4_{Mite} (Der p 1) (**b**) were measured before, after 8 weeks and after 16 weeks of a classic SIT treatment (●) or a cluster SIT (■). Data are presented as single values and median.

week 3: 38.3 µg/l, 6.2–119). In the classic group a significant decline was found after 16 weeks (week 1: 43.6 µg/l, 8.5–201; week 2: 57.2 µg/l, 10.5–173; week 3: 33.4 µg/l, 11.1–71.5, $p < 0.05$). Comparison between the two groups showed a significant difference after 8 weeks (V2, $p < 0.01$).

Specific Antibody Response

Immunogenicity of the different treatment regimes was determined by specific antibodies against Der p 1, IgE_{Mite}, IgG_{Mite}, and IgG4_{Mite} in patient serum during the treatment period (week 1, 8 and 16). Specific IgE_{Mite} con-

centrations increased significantly at week 8 in the cluster group (week 1: 106 kU/ml, 12.0–593; week 8: 184 kU/ml, 13.3–612; week 16: 198 kU/ml, 11.0–536, $p < 0.05$), but not in the classic treatment regime (week 1: 95.8 kU/ml, 10.0–1,325; week 8: 92 kU/ml, 9.2–1,307; week 16: 135 kU/ml, 11.7–1,678).

Specific IgG_{Mite} (week 1: 14.3 kU/ml, 8.4–30.8; week 8: 30.7 kU/l, 20.0–83.9, $p < 0.001$; week 16: 36.6 kU/ml, 23.9–83.6) and specific IgG4_{Mite} (week 1: 0.35 kU/ml, 0.35–14.8; week 8: 7.9 kU/l, 0.3–84.0, $p < 0.001$; week 16: 17.1 kU/ml, 5.8–75.8, $p < 0.05$) increased significantly after 8 weeks in the cluster group. In contrast, both parameters (spe-

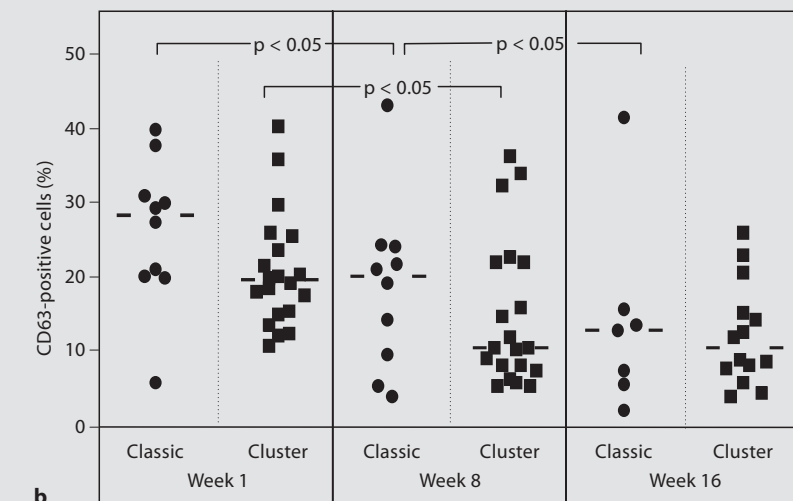
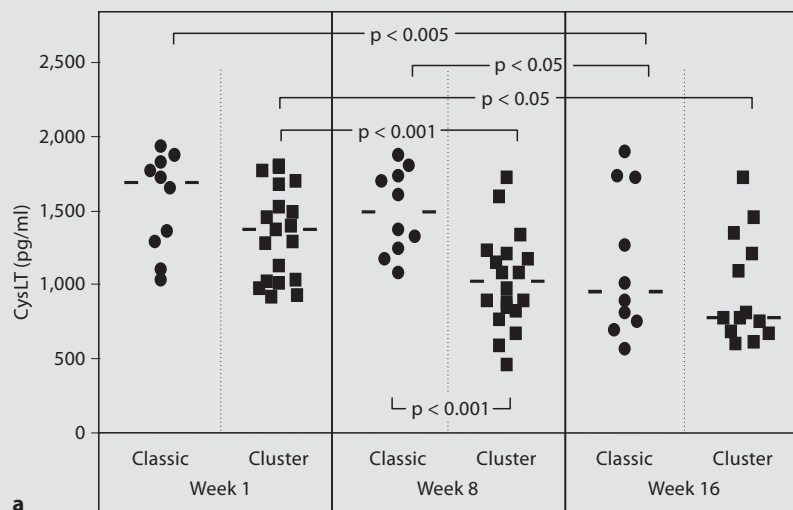


Fig. 2. Blocking antibodies derived from patients treated with classic SIT treatment (●) or a cluster SIT (■) were measured before, after 8 weeks and after 16 weeks of the study. The inhibitory properties of the antibodies were detected by cysLT release (a) and CD63 expression (b) of in vitro mite-stimulated basophils. Data are presented as single values and median.

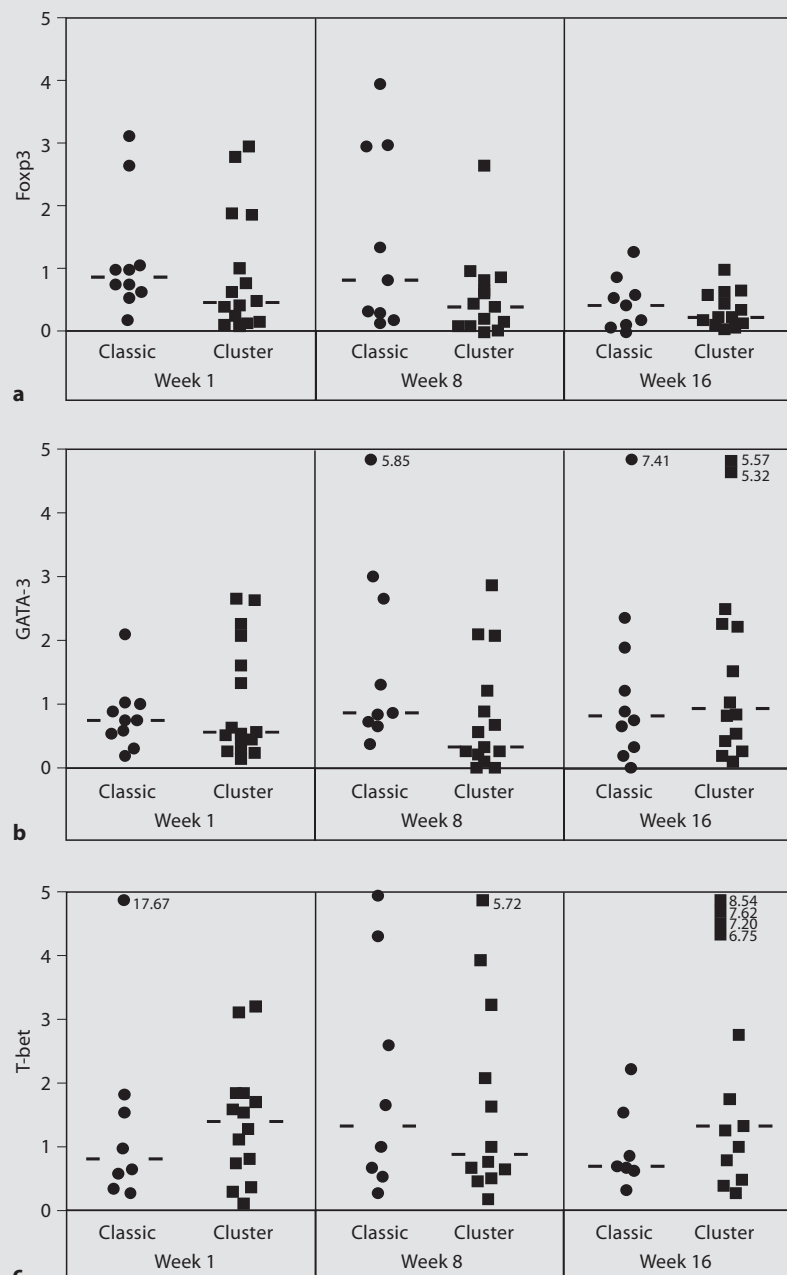
cific IgG_{Mite} week 1: 11.3 kU/ml, 8.7–20.2; week 8: 14.0 kU/l, 10.1–41.1; week 16: 32.5 kU/ml, 23.6–84.8, $p < 0.05$; specific IgG_{4Mite} week 1: 0.35 kU/ml, 0.35–4.1; week 8: 0.35 kU/l, 0.35–1.9; week 16: 10.6 kU/ml, 0.35–89.1, $p < 0.05$) reached significance not before 16 weeks of treatment in the classic SIT group (fig. 1). Comparison between the two groups showed significant differences of specific IgG_{Mite} ($p < 0.001$) as well as specific IgG_{4Mite} ($p < 0.001$) concentrations after 8 weeks of treatment. Similar results were found for antibody titers against Der f 1 (data not shown).

Blocking Antibodies

We tested the inhibitory properties of serum antibodies by the activation of basophils in order to inhibit cysLT secretion and CD63 expression.

In vitro stimulation with house dust mite extract showed a striking cysLT release in both groups at baseline that became significantly lower in the cluster group (week 1: 1,377 pg/ml, 319–1,796; week 8: 1,021 pg/ml, 461–1,712; week 16: 786 pg/ml, 590–1,888, $p < 0.001$) after 8 weeks compared to the classic group, which reached a significant decrease of cysLT not before week 16 (week 1: 1,518 pg/ml, 1,025–1,929; week 8: 1,280 pg/ml, 753–1,800; week

Fig. 3. Foxp3 (a), GATA-3 (b) and T-bet (c) mRNA expression from separated T cells were analyzed by a specific real-time PCR strategy. Transcription factors were measured before, after 8 weeks and after 16 weeks in patients treated with a classic SIT (●) or a cluster SIT (■) and expressed as relative values compared to a respective standard. Data are presented as single values and median.



16: 845 pg/ml, 557–1,887, $p < 0.005$) (fig. 2a). Between-group comparison showed a significantly lower cysLT release in the cluster regime after 8 weeks ($p < 0.001$).

In contrast, mite dust extract-induced CD63 expression significantly decreased in the cluster group (week 1: 19.65%, 10.84–40.37; week 8: 10.58%, 5.33–36.27; week 16: 10.48%, 3.92–25.97, $p < 0.05$) as well as in the classic

group (week 1: 28.48%, 5.88–39.87; week 8: 20.17%, 3.92–43.21; week 16: 13.07%, 2.01–41.63, $p < 0.05$) after 8 weeks compared to baseline (fig. 2b). However, whilst the cluster group reached its plateau at week 8, CD63 expression went further down at week 16 in the classic group ($p < 0.05$).

T Cell Subset Transcription Factors

The capacity of the different treatment regimes on the expression of T cell subset transcription factors was analyzed by using real-time PCR strategy. Neither within the groups nor between the treatment regimes were any differences in Foxp3, T-bet, or GATA-3 expression found (fig. 3).

Discussion

In the present study, we demonstrated that a cluster schedule for the induction phase of immunotherapy is as safe as the classic regime in children, allowing administration of high allergen doses in a short time interval compared to conventional SIT. In addition to this, we studied different immune parameters including T cell transcription factors to get further insights into early immunological changes.

Looking at the safety data, there were no significant differences in local and systemic reactions comparing the two schedules. We administered 492 doses of allergen to 30 patients, with 0.6% of non-life-threatening grade 3 systemic reactions per dose in the cluster group and 0.7% in the classic group. A very important point especially in our pediatric study was the absence of severe systemic reactions. However, we started our study with a cluster schedule adapted from a cluster SIT in adults who suffered from seasonal allergic rhinitis [22]. This cluster regime is now licensed for adults with allergic rhinitis and asthma in Germany and this was one of the reasons why our ethics committee gave approval for the study. Surprisingly, we recorded an increase of systemic side effects (mainly coughing) in 5 patients in the cluster group after receiving a total dose of 1.2 ml (6,000 TU) at week 4. Therefore, we modified the cluster regimen due to safety concerns as follows: at week 4, only two injections (a total of 0.6 ml = 3,000 TU) were given not exceeding the maintenance dose of 1 ml (5,000 TU). Changing the cluster protocol at week 4 resulted in a clear decrease of systemic side effects. This clear-cut difference compared to the study which used the same protocol in adults is difficult to explain. The main reason may be that we studied a highly selected group of children who all suffered from bronchial asthma allergic to mites proven by bronchial allergen challenge. Moreover, it is well known that side effects in allergic asthma are higher compared to allergic rhinitis [23].

To our knowledge, allergic inflammation has not been previously used as an outcome measure in a cluster im-

muno-therapy study. Allergic inflammation was assessed by using eNO and measurement of ECP. Both parameters, eNO and ECP, are a useful markers to monitor allergen exposure, degree of allergic inflammation and effects of anti-inflammatory therapy [24–27].

Interestingly, we found a more rapid decrease of both markers in the cluster group compared to the classic up-dosing schedule. However, we have to admit that the decrease of eNO failed to reach significance, most likely because eNO levels were already suppressed by inhaled corticosteroids in some of the patients.

Looking at the specific immunological effects, we observed a rapid and highly significant increase in mite-specific IgE, IgG, and IgG4 in the cluster group compared to the classic regime. These differences were equalized at week 16 and may not be relevant with respect to the clinical effects. Immunotherapy with inhalant allergens is associated with increases in serum allergen-specific IgG and IgG4 levels [28]. IgG antibodies have been proposed to act as blocking antibodies by competing with IgE for allergen binding to mast cells, basophils, and other IgE receptor-expressing cells [29]. IgG4 induced by means of immunotherapy blocks allergen-induced IgE-dependent histamine release by basophils and suppresses allergen-specific T cell responses in vitro by inhibiting binding of IgE allergen complexes to antigen-presenting cells [30]. This is in keeping with our functional findings on allergen-induced mediator release. Clearly, the release of cysLT in whole blood was downregulated and CD63 expression on basophils was inhibited to some extent during the SIT in both treatment regimes.

There are contradictory reports on the role of IgG in the efficacy of the immunotherapy [31, 32]. The issue of whether there is a cause-and-effect relationship between the increase in IgG levels and the symptomatic improvement conferred by immunotherapy or whether this is just a surrogate marker of exposure to the SIT remains unresolved. However, higher IgG concentrations are independently associated with symptomatic improvement in many studies, implying a close linkage between immunological and clinical changes [8, 16].

Another attractive hypothesis for the immunological basis of SIT is its effects on Treg cells [33]. Treg cells are able to inhibit the development of allergic TH2 responses and play a major role in allergen SIT [34, 35].

Therefore, we also attempted to study the effect of both treatment regimes on the balance of the T cell subset transcription factors for Treg cells (Foxp3), TH1 cells (T-bet), and TH2 cells (GATA-3) in children. Our study shows no significant changes in the distribution of all the

transcription factors. Our findings seem to contradict the results of some previous studies investigating the effect of immunotherapy on the induction of Treg cells. Jutel et al. [19] showed that T cell suppression observed in the course of venom immunotherapy is induced by Treg cells with high IL-10 and TGF- β induction. It was suggested that inhibition of IgE and enhancement IgG4 production induced by Treg cells may actually be one of the main mechanisms underlying the clinical effects of immunotherapy.

It is possible that age is one of the factors underlying the discrepant results and accounting for the different results of our study in children. Alternatively, the differences in the type of allergen (venom vs. mite) and the type of immunotherapy (rush vs. cluster/classic) might be important in the determination of Treg cells and their function. However, precise factors underlying the discrepant results among these studies remain to be elucidated.

Our study has some important limitations including the change of protocol after 5 patients, the lack of a placebo group, and the open trial design. The injection of a placebo is generally unacceptable in pediatric populations and was considered to be unethical. In addition, because patients with a positive bronchial allergen provocation test were considered to be ideal candidates for SIT, we felt that it would be inappropriate to withhold SIT from these patients.

The consistency of laboratory findings and the clear-cut differences observed between the cluster and classic immunotherapy during the induction phase seems to be of value for further convenient concepts of SIT for parents and children.

Interestingly, despite the clear beneficial effect of immunotherapy in allergic rhinitis and asthma, only a few allergic patients subscribe to this treatment [36]. Inconvenience is likely the reason for not choosing this treatment and one of the most frequent reasons for discontinuing it [37, 38]. This inconvenience is primarily due to the time involved, because it is recommended that specific allergen immunotherapy be administered in a medical facility. As shown, even in children accelerated immunotherapy induction schedules may provide a rapid alternative to conventional up-dosing schedules without a significant increase in risk but an increased compliance with a reasonable therapy strategy.

In conclusion, these data demonstrate that (1) a cluster SIT is safe and well-tolerated in children suffering from house dust mite asthma, (2) serum allergen-specific IgG and IgG4 titers are induced more rapidly, and (3) it is less time-consuming for patients as compared with the classic SIT.

References

- 1 Passalacqua G, Durham SR: Allergic rhinitis and its impact on asthma update: allergen immunotherapy. *J Allergy Clin Immunol* 2007;119:881–891.
- 2 Finegold I: Allergen immunotherapy: present and future. *Allergy Asthma Proc* 2007;28:44–49.
- 3 Bousquet J, Lockey R, Malling HJ: Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. *J Allergy Clin Immunol* 1998;102:558–562.
- 4 Malling HJ: Immunotherapy as an effective tool in allergy treatment. *Allergy* 1998;53:461–472.
- 5 Durham SR, Walker SM, Varga EM, Jacobson MR, O'Brien F, Noble W, Till SJ, Hamid QA, Nouri-Aria KT: Long-term clinical efficacy of grass-pollen immunotherapy. *N Engl J Med* 1999;341:468–475.
- 6 Klimek L, Dormann D, Jarman ER, Cromwell O, Riechelmann H, Reske-Kunz AB: Short-term preseasonal birch pollen allergoid immunotherapy influences symptoms, specific nasal provocation and cytokine levels in nasal secretions, but not peripheral T-cell responses, in patients with allergic rhinitis. *Clin Exp Allergy* 1999;29:1326–1335.
- 7 Klimek L, Malling HJ: Specific immunotherapy (hyposensitization) in allergic rhinoconjunctivitis. Meta-analysis of effectiveness and side effects (in German). *HNO* 1999;47:602–610.
- 8 Tabar AI, Echechipia S, Garcia BE, Olaguibel JM, Lizaso MT, Gomez B, Aldunate MT, Martin S, Marcotegui F: Double-blind comparative study of cluster and conventional immunotherapy schedules with *Dermatophagoides pteronyssinus*. *J Allergy Clin Immunol* 2005;116:109–118.
- 9 Mauro M, Russello M, Alesina R, Sillano V, Alessandrini A, Dama A, Passalacqua G, Senna G: Safety and pharmacoeconomics of a cluster administration of mite immunotherapy compared to the traditional one. *Eur Ann Allergy Clin Immunol* 2006;38:31–34.
- 10 Tabar AI, Muro MD, Garcia BE, Alvarez MJ, Acero S, Rico P, Olaguibel JM: *Dermatophagoides pteronyssinus* cluster immunotherapy. A controlled trial of safety and clinical efficacy. *J Investig Allergol Clin Immunol* 1999;9:155–164.
- 11 Moreno C, Fernandez-Tavora L, Acero S, Alonso MD, Barahona MJ, Blanco R, Cisteró A, Conde J, Fernández J, Fernández S, Fernández-Rivas M, García BE, García-Rodríguez R, Camacho E, González-Quevedo T, Gonzalo A, Guardia P, Sánchez-Cano M, Tabar AI, de la Torre F: Tolerance of a cluster schedule on the treatment of seasonal allergic respiratory disease with pollen extracts quantified in mass units. *J Investig Allergol Clin Immunol* 2003;13:221–227.
- 12 Tabar AI, Fernandez-Tavora L, Alonso R, Castillo R, Cistero-Bahima A, de la Torre-Morin F, Fernández J, García-Figueroa BE, Fernández S, García-González JJ, García-Robaina JC, Moreno F, Lobatón P, Sánchez-Machín I, de la Torre-Martínez F: Tolerance of a cluster schedule with a house dust mite extract quantified in mass units: multicentre study. *J Investig Allergol Clin Immunol* 2004;14:193–197.

- 13 Durham SR, Till SJ: Immunologic changes associated with allergen immunotherapy. *J Allergy Clin Immunol* 1998;102:157–164.
- 14 Rossi RE, Monasterolo G, Coco G, Silvestro L, Operti D: Evaluation of serum IgG4 antibodies specific to grass pollen allergen components in the follow up of allergic patients undergoing subcutaneous and sublingual immunotherapy. *Vaccine* 2007;25:957–964.
- 15 Garcia BE, Sanz ML, Gato JJ, Fernandez J, Oehling A: IgG4 blocking effect on the release of antigen-specific histamine. *J Investig Allergol Clin Immunol* 1993;3:26–33.
- 16 Nouri-Aria KT, Wachholz PA, Francis JN, Jacobson MR, Walker SM, Wilcock LK, Staple SQ, Aalberse RC, Till SJ, Durham SR: Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. *J Immunol* 2004;172:3252–3259.
- 17 Jutel M, Pichler WJ, Skrbic D, Urwyler A, Dahinden C, Muller UR: Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN-gamma secretion in specific allergen-stimulated T cell cultures. *J Immunol* 1995;154:4187–4194.
- 18 Francis JN, Till SJ, Durham SR: Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol* 2003;111:1255–1261.
- 19 Jutel M, Akdis M, Budak F, Aebischer-Casaulta C, Wrzyszc M, Blaser K, Akdis CA: 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;33:1205–1214.
- 20 Cockcroft DW, Killian DN, Mellon JJ, Hargreave FE: Bronchial reactivity to inhaled histamine: a method and clinical survey. *Clin Allergy* 1977;7:235–243.
- 21 Rosewich M, Rose MA, Eickmeier O, Travaçi M, Kitz R, Zielen S: Montelukast as add-on therapy to beta-agonists and late airway response. *Eur Respir J* 2007;30:56–61.
- 22 Hansen I, Hormann K, Stuck BA, Schneider-Gene S, Mosges R, Klimek L: Cluster-immunotherapy in seasonal allergic rhinitis: safety aspects of induction therapy with depot allergoids (Purethal) (in German). *Laryngo-rhinootologie* 2003;82:558–563.
- 23 Halken S: Prevention of allergic disease in childhood: clinical and epidemiological aspects of primary and secondary allergy prevention. *Pediatr Allergy Immunol* 2004;15:4–5, 9–32.
- 24 Barnes PJ, Liew FY: Nitric oxide and asthmatic inflammation. *Immunol Today* 1995;16:128–130.
- 25 Ohashi Y, Nakai Y, Kakinoki Y, Ohno Y, Sakamoto H, Kato A, Tanaka A: Effect of immunotherapy on serum levels of eosinophil cationic protein in perennial allergic rhinitis. *Ann Otol Rhinol Laryngol* 1997;106:848–853.
- 26 Bez C, Schubert R, Kopp M, Ersfeld Y, Rosewich M, Kuehr J, Kamin W, Berg AV, Wahn U, Zielen S: Effect of anti-immunoglobulin E on nasal inflammation in patients with seasonal allergic rhinoconjunctivitis. *Clin Exp Allergy* 2004;34:1079–1085.
- 27 Cevit O, Kendirli SG, Yilmaz M, Altintas DU, Karakoc GB: Specific allergen immunotherapy: effect on immunologic markers and clinical parameters in asthmatic children. *J Investig Allergol Clin Immunol* 2007;17:286–291.
- 28 Keskin O, Tuncer A, Adalioglu G, Sekerel BE, Sackesen C, Kalayci O: The effects of grass pollen allergoid immunotherapy on clinical and immunological parameters in children with allergic rhinitis. *Pediatr Allergy Immunol* 2006;17:396–407.
- 29 Wachholz PA, Durham SR: Induction of 'blocking' IgG antibodies during immunotherapy. *Clin Exp Allergy* 2003;33:1171–1174.
- 30 van Neerven RJ, Wikborg T, Lund G, Jacobsen B, Brinch-Nielsen A, Arnved J, Ipsen H: Blocking antibodies induced by specific allergy vaccination prevent the activation of CD4+ T cells by inhibiting serum-IgE-facilitated allergen presentation. *J Immunol* 1999;163:2944–2952.
- 31 Bousquet J, Maasch H, Martinot B, Hejjaoui A, Wahl R, Michel FB: Double-blind, placebo-controlled immunotherapy with mixed grass-pollen allergoids. II. Comparison between parameters assessing the efficacy of immunotherapy. *J Allergy Clin Immunol* 1988;82:439–446.
- 32 Wachholz PA, Soni NK, Till SJ, Durham SR: Inhibition of allergen-IgE binding to B cells by IgG antibodies after grass pollen immunotherapy. *J Allergy Clin Immunol* 2003;112:915–922.
- 33 Larche M: Regulatory T cells in allergy and asthma. *Chest* 2007;132:1007–1014.
- 34 Bellinghausen I, Metz G, Enk AH, Christmann S, Knop J, Saloga J: Insect venom immunotherapy induces interleukin-10 production and a Th2-to-Th1 shift, and changes surface marker expression in venom-allergic subjects. *Eur J Immunol* 1997;27:1131–1139.
- 35 Marcotte GV, Braun CM, Norman PS, Nicodemus CF, Kagey-Sobotka A, Lichtenstein LM, Essayan DM: Effects of peptide therapy on ex vivo T-cell responses. *J Allergy Clin Immunol* 1998;101:506–513.
- 36 Abramson MJ, Puy RM, Weiner JM: Is allergen immunotherapy effective in asthma? A meta-analysis of randomized controlled trials. *Am J Respir Crit Care Med* 1995;151:969–974.
- 37 Cohn JR, Pizzi A: Determinants of patient compliance with allergen immunotherapy. *J Allergy Clin Immunol* 1993;91:734–737.
- 38 Lower T, Henry J, Mandik L, Janosky J, Friday GA Jr: Compliance with allergen immunotherapy. *Ann Allergy* 1993;70:480–482.