Allergoid-specific T-cell reaction as a measure of the immunological response to specific immunotherapy (SIT) with a Th1-adjuvanted allergy vaccine

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Summary. *Background:* Specific immunotherapy (SIT) is believed to modulate CD4+ T-helper cells. In order to improve safety, SIT vaccines are often formulated with allergoids (chemically modified allergens). Interaction between T-cells and allergoids is necessary to influence cellular cytokine expression. There have been few reports on identification the early cellular effects of SIT.
Method: Patients allergic to grass and/or mugwort pollen (n= 21) were treated with a 4-shot allergy vaccine (Pollinex Quattro) containing appropriate allergoids (grass/rye and/or mugwort) adsorbed to L-tyrosine plus a Th1 adjuvant, monophosphoryl lipid A (MPL®). Fourteen grass-allergic patients served as untreated controls. Using the peripheral blood mononuclear cells of these patients, an optimized lymphocyte transformation test (LTT) was employed to monitor the *in vitro* proliferative response of T-cells to an allergoid challenge (solubilised Pollinex Quattro) before the first and last injection and then 2 and 20 weeks after the final injection. Control challenges utilised preparations of a similar pollen vaccine without the adjuvant MPL® and a tree pollen vaccine with and without MPL®.
Results: The LTT showed increased LTT stimulation indices (SI) in 17/20 SIT patients when the solublised vaccine preparation was used as a challenge before the last injection and 2 weeks after, in comparison to pre-treatment levels. Twenty weeks after therapy, the SI decreased to baseline level. A vaccine challenge without MPL® gave lower SI levels. A challenge of a clinically inappropriate tree allergoid vaccine gave no response, and a non-treated group also showed no response.
Conclusion: Following a short-course SIT adjuvated with MPL®, challenges of allergoids were shown to activate allergen-specific T cells *in vitro*. There was an additional stimulating effect when the challenge was in combination with MPL®. There were no non-specific effects of MPL®, shown by the tree allergoid/ MPL® control. The timing of the response was closely correlated to the treatment course; reactivity fell two weeks after the final injection and 20 weeks later it was at baseline level. Thus an immunological response to SIT was detected after very few injections. This methodology could provide a basis for monitoring the immediate progress of allergy vaccinations.

Key words: Lymphocyte transformation, T cells, SIT, allergoid, tree, grass, monophosphoryl lipid A, MPL®

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Introduction

Successful Specific immunotherapy (SIT) is believed to modulate CD4+ helper cells, resulting in a reduced activation of inflammatory cells, a down-regulation of mast-cell activity and a reduced basophil histamine release [1]. SIT also influences early and late-phase reactions after allergen provocation. It is also well known that SIT leads to an eventual reduction in proliferation to allergen challenge *in vitro*. These changes, usually only observed after many months, presumably stem from the patient's allergen-specific T-cell reaction resulting from the applied allergens or allergoids. In this study of patients with grass pollen allergy, the examination of the T-cell reactivities to challenges of the therapeutic SIT preparations could probably give an early indication of their individual immunological responses.

Antigen-induced T-cell responsiveness is commonly assayed *in vitro* by measuring the proliferative response of primed T lymphocytes to antigenic challenge. The lymphocyte transformation test (LTT) is a well-established *in vitro* method for investigating antigen-specific cellular immune responses. The proliferative response of cultured lymphocytes is measured by quantifying the uptake of tritium-labelled thymidine (^{3}H-thymidine) into the DNA of antigen-activated T cells. In this study we used a recently optimised LTT, in which the methodology was improved by the addition of recombinant interferon alpha (rIFN-α) to the cell culture. rIFN-α facilitates enhanced antigen presentation of monocytes by reinforcing the expression of the surface molecules involved and also suppresses the proliferation of non-specifically activated lymphocytes by its known antiproliferative properties [2].

This paper describes the monitoring of the T-cell reaction to allergoid preparations in patients undergoing a short injection course of SIT. Although native allergens may have been used for the challenge, it was decided to use the active components of the vaccine itself. This approach was relatively novel, but certainly appropriate in order to mirror the vaccine antigens. The therapy consisted of four subcutaneous injections of a standardized allergy vaccine comprising a suspension of an L-tyrosine-adsorbed allergoid pollen extract. This has been reported as efficacious despite using only four pre-seasonal injections [3].

Patients and Methods

Patients

Sixteen patients were investigated in a preliminary study to ascertain the applicability of the LTT for this investigation. Nine of these patients suffered from pollinosis to grass pollens and the remaining seven were allergic to tree pollens, particularly birch, alder and hazel (but excluding grass pollens). Diagnosis was established by clinical confirmation and skin prick test.

The follow-up study comprised 35 patients who were allergic to grass and/or mugwort pollen and identified for inclusion by allergy diagnosis in a general practice. The diagnosis was confirmed by clinical history of seasonal allergic rhinitis, positive skin prick test sensitivity (diagnostic test solutions, Bencard/Allergy Therapeutics, Munich, Germany) and allergen-specific IgE detection (Ridascreen®, Biopharm, Darmstadt, Germany). SIT was administered to 21 patients (9 male, 11 female, aged 18-61, mean age 33 years) following the study protocol. The untreated control group consisted of 14 patients (6 male, 8 female, aged 18-44, mean age 29 years). Assignment to the groups was random. The two groups of patients were not different with regard to disease severity, skin test or specific IgE values, age, or sex distributions. All patients gave their informed consent to the study.

The 21 SIT-treated patients received four injections of a standardised allergoid vaccine prior to the pollen season, at one-weekly intervals with increasing dosage of 300, 800, 2 x 2000 Standardised Units (SU)/ml. The vaccine was composed of glutaraldehyde-modified pollen extracts adsorbed to L-tyrosine in an aqueous suspension [3]. Allergoids that were used according to the individual sensitivities of patients comprised: grasses, n= 9; mugwort n= 7; grasses/mugwort n= 5. This formulation (Pollinex® Quattro, Bencard/Allergy Therapeutics, Germany) also contained monophosphoryl lipid A (MPL® adjuvant). MPL® is a detoxified lipopolysaccharide component extracted and purified from *Salmonella minnesota* [4].

The 14 patients of the control group received symptomatic therapy as required, with levocabastin eye drops and nasal spray (Livocab-Kombi ®, Janssen-Cilag, Neuss, Germany) and cetirizine (Zyrtec ®, UCB Pharma, Kerpen, Germany). As the therapy period was before the grass pollen season, the drug treatment was only needed in isolated cases.

Study Protocol

Heparinized blood for the *in vitro* experiments was drawn from the cubital vein of all 35 patients at 4 time points: before the 1st injection, before the 4th (last) injection, two weeks after the last injection and at the end of the pollen season (5 months after the last injection).

Lymphocyte transformation test

Peripheral blood mononuclear cells (PBMCs) from the heparinized venous blood of the patients were isolated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation within four hours after venipuncture. After rinsing the cells twice with

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phosphate buffered saline (PBS), (Sigma, Deisenhofen, Germany) the cell pellet was resuspended at a concentration of 1.5 x 10^6 cells/ml in RPMI 1640 culture medium (Biochrom, Berlin, Germany), supplemented with 2 mM L-glutamine (Sigma), 100 µg/ml gentamycin (Seromed, Heidelberg, Germany) and 5% autologous serum. All the cultures were started in triple sets with a final volume of 200µl (3 x 10^5 cells/ml) in 96-well flat bottom microtitre plates (Nunclon, Wiesbaden, Germany). The lymphocyte transformation was analysed after a 6-day culture (37°C, 5% CO_2) by incorporating ^{3}H-thymidine (Amersham, Great Britain) into the newly formed DNA. The radioactive marker (1 µCi/ml) was added to the culture for the final 12 hours of culture. Cells were harvested onto glass fibre filters (Wallac, Lund, Sweden) and the incorporated ^{3}H-thymidine activity was determined as counts per minute (cpm) using a ß-counter (Wallac). The results were reported as total activity (cpm) and as ratio between stimulated and non-stimulated proliferation (stimulation index, SI). A stimulation index > 3 was considered to be positive.

Modification of the standard lymphocyte transformation test

20µl recombinant human interferon-α 2a (Biosource, Giessen, Germany) stock solution prediluted in RPMI to1250 IU/ml was added to the plated cells immediately before adding the appropriate antigen. The resulting final concentration of IFN-α in the culture was 125 IU/ml. Our preliminary experiments using increasing doses from 10 to 10,000 IU/ml IFN-α indicated this concentration as optimal for enhancing the stimulation index without obtaining false positive or negative results [2].

Antigenic stimulants used in the LTT

For allergen-specific stimulation we used a simple dilution of the vaccines (L-tyrosine suspensions containing adsorbed allergoids) as used in the immunotherapy treatment. This simple procedure provides totally soluble allergoids at a dilution of 1:100 (the solubility of L-tyrosine is 450mg/Litre at neutral pH). Lower dilutions were also examined to observe the possible activity of surface-adsorbed allergoids. Cells from all 35 patients of the therapy and control groups were challenged with the four allergoid preparations. Firstly, the therapeutically applied Pollinex® Quattro grass/rye vaccine (optionally with mugwort) containing the adjuvant MPL®. Secondly, the corresponding vaccine without MPL adjuvant (TA Mix). Thirdly, the clinically innapropriate tree pollen vaccine containing MPL® (Pollinex® Quattro birch/alder/hazel). Fourthly, the corresponding vaccine without MPL adjuvant (TA Mix Baumpollen).

Preliminary experiments using these vaccines at a wide range of dilutions from 1:2 to 1:10000 revealed that the most appropriate dilution for stimulation was a 1:100 (v/v) dilution for all four vaccines. At this level, inappropriate components (i.e. excipients) in the vaccines are also diluted to levels where they are unlikely to interfere with the LTT test.

Cultures from each patient were stimulated with the recall antigen Purified Protein Derivative (PPD) to provide positive controls. The test results were considered valid when these cultures showed a SI of 10 or higher with a PPD challenge.

Statistical analysis

Results are given as means (± SEM). Differences in the stimulation indices of the patients between the time points were evaluated for significance using the Wilcoxon test for paired samples. Differences between groups were evaluated for significance using the Mann-Whitney U-test. Results were considered statistically significant at the $p = 0.05$ level.

Results

Clinical outcomes

Symptom scores (eyes, nose, lungs) from all patients were totalled and compared to those found in the preceding pollen season. The pollen load in both years was comparable. In the SIT group, all patients (21/21) showed improved symptoms. In the control group, no improvements were observed (0/14). The persistence of the symptoms within the control group and consequent need for rescue medication during the season encouraged 11 patients to be treated with the SIT used in this study as prophylaxis for the following season.

Suitability of therapeutic vaccines in providing an antigen challenge for investigating specific allergoid-induced lymphocyte proliferation

Cells from 9 non-treated patients allergic to grass pollens were separately challenged with Pollinex® Quattro grass/rye and TA Mix Gräserpollen using dilutions of 1:10, 1:100, 1:1000 and 1:10000. Allergoids from both of these vaccines induced significant dose-dependent lymphocyte activation in 7 of 9 patients. Two patients showed no *in vitro* T cell response independently of the stimulant. In 6 of 7 cases with a positive response, the optimum dilution was a final dilution of 1:100. Thus a 1:100 concentration was chosen for all the following

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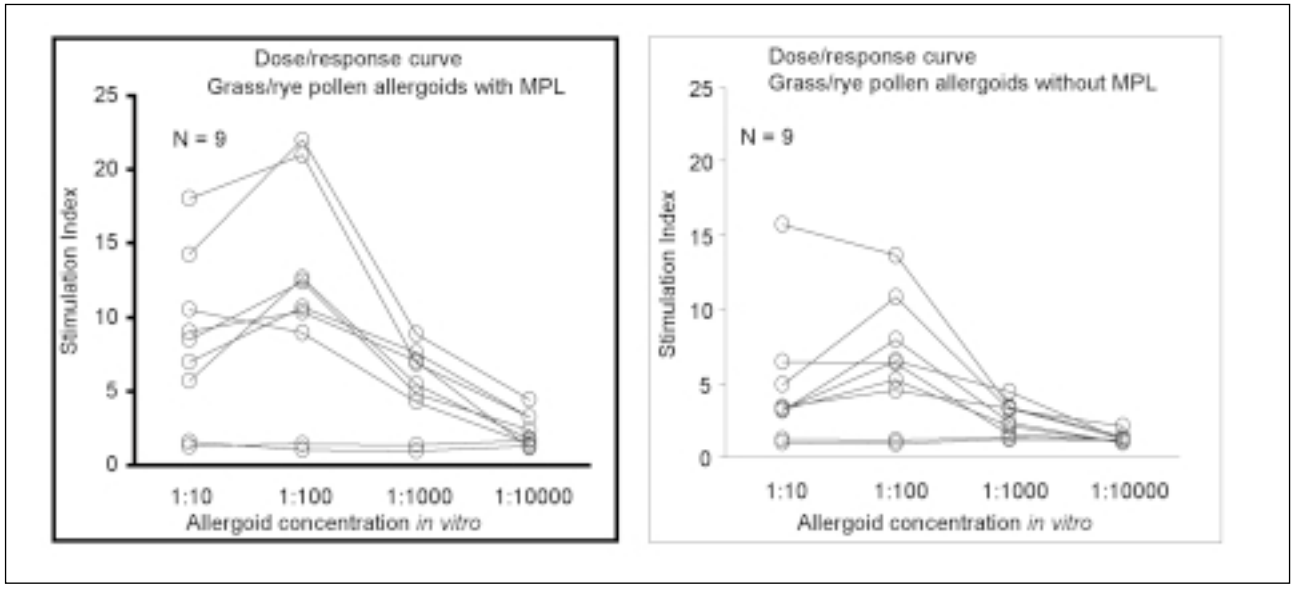

Figure 1. Dose-dependent lymphocyte activation *in vitro* using grass/rye allergoids with MPL [left] or grass/rye allergoids without MPL [right] using final dilutions of L-tyrosine adsorbate suspensions at 1:10, 1:100, 1:1000 and 1:10000 v/v added to cells from 9 grass-pollen allergic patients.
The stimulation index is the quotient derived from the allergen-induced lymphocyte response and the non-stimulated lymphocyte response.

LTT investigations. The dose response curves are illustrated in Figure 1.

The specificity of lymphocyte proliferation was tested using cells from 7 grass pollen-allergic patients known not to be allergic to tree pollen and challenged with grass and tree pollen allergoids in parallel. The specificities were clearly confirmed by the high indices for grass antigens and the negative response for tree antigens as shown in Figure 2. *Vice versa*, in a similar experiment cells from 7 subjects with tree pollen allergy (but not grass pollen allergy) responded well to the tree pollen antigens and poorly to the grass pollen antigens (Figure 2).

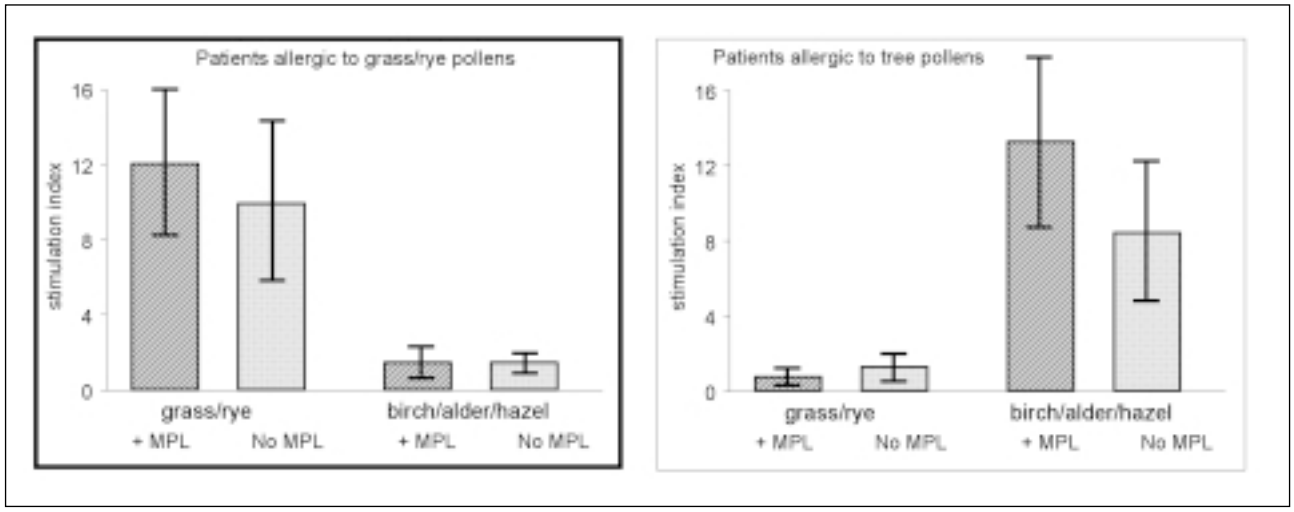

Figure 2. Specificity of *in vitro* lymphocyte activation. Left: stimulation of cells from patients allergic to grass pollens using challenges of grass or tree pollen allergoids. Right: stimulation of cells from patients allergic to tree pollens using challenges of grass or tree allergoids. Mean values +/- SEM of the stimulation indices of 7 patients in each group. Allergoid/tyrosine dilution in all preparations = 1:100 v/v.

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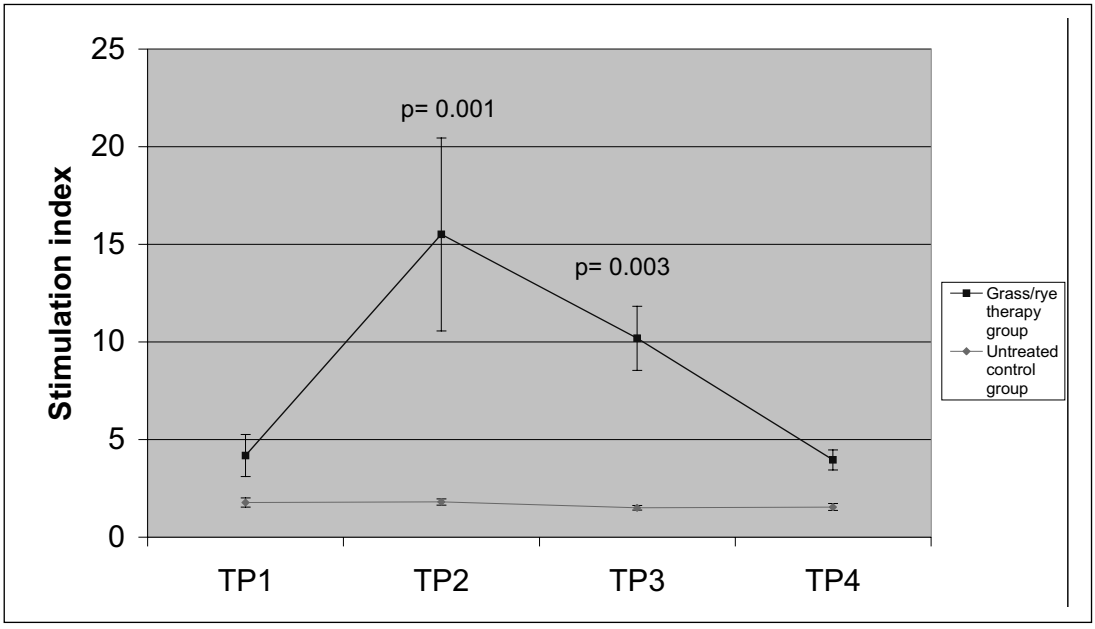

Figure 3. Comparison of stimulation indices for grass/rye group compared to the untreated control group.

Effect of on-going SIT on stimulation indices using the therapeutic allergoid as stimulant

Before treatment initiation, cells from 9 of the 20 subjects in the therapy group displayed a positive response (SI >3) to the therapeutic allergoid preparation (Pollinex Quattro® grass/rye and/or mugwort). However, prior to the fourth SIT injection 20 out of 21 patients had indices of > 3 with this challenge. In 17 of 21 patients the SI was increased when compared to the initial value. This correlates with a significant increase in the mean stimulation index (SI = 16.35 ± 4.49, mean ± S.E.M), (p = 0.001) in comparison to initial values (SI = 4.41 ± 1.1)(Fig. 3) Two weeks after the final injection, the mean SI was still significantly higher compared to the pre-treatment level (SI = 10.18 ± 1.7, p = 0.003, 17/20). The mean stimulation index after 5 months was below the initial level (SI = 3.95 ± 0.5) though this did not quite reach significance.

In the non-treated control group at no time in the study was there a significant change in the mean stimulation index (mean ± SEM TP1: 1.77 ± 0.24; TP2: 1.8 ± 0.16; TP3: 1.5 ± 0.12; TP4: 1.54 ± 0.19) as shown in Fig. 3.

Effect of a control tree pollen allergoid challenge on lymphocytes from patients insensitive to tree pollen

A subgroup (n=14) of the treated patients was investigated with the control allergoid Pollinex Quattro birch/alder/hazel. We found no significant alterations of the stimulation indices at any time point. Four of these patients gave a positive response (SI > 3) but there were no significant alterations when compared to the starting level timepoint (TP) 1 (mean ± SEM TP1: 1.76 ± 0.39; TP2: 2.23 ± 0.41; TP3: 2.18 ± 0.38; TP4: 2.22 ± 0.67). Furthermore, cells from an untreated control group showed no significant alterations over time when challenged with the same antigen (data not shown).

Comparison of cell reaction to allergoids with and without MPL adjuvant

Because it was possible that the MPL® adjuvant from the Pollinex Quattro vaccine could have non-specific activation effects in the cell culture, the MPL® -free TA Mix Gräserpollen and/or mugwort and TA Mix Baumpollen allergoid vaccines were tested in parallel at all times. In general, none of the 35 patients showed a different result in relation to an existing or non-existent cellular sensitisation. When comparing the responses to antigen with and without MPL® adjuvant, the SI values using the adjuvant containing vaccine (Pollinex Quattro) were higher at time points 2 and 3 (TP2: 16.35 ± 4.92 vs. 11.1 ± 2.49, n.s.; TP3: 10.18 ± 1.69 vs. 4.88 ± 0.97, p = 0.002). Furthermore, the mean stimulation index in the latter group was also significantly increased two weeks after the final injection (TP3). At this timepoint the SI values from the MPL® -free challenge group had nearly reached baseline again. These comparisons are illustrated in Figure 4.

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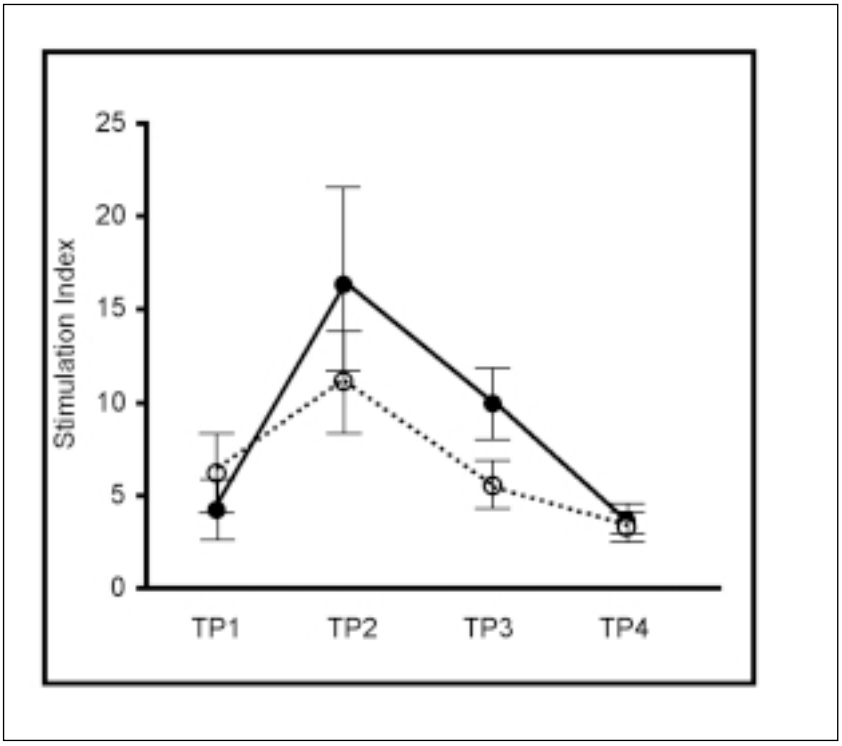

Figure 4. Comparison of cells from the active therapy group stimulated with grass/rye (mugwort) allergoid with and without MPL adjuvant (n=Mean ± S.E.M.). Allergoid/MPL: full line. Allergoid alone: dotted line. Significances over timepoint 1: Allergoid/MPL, TP2 p=0.001; TP3 p=0.003. Allergoid alone, TP2 p<0.01; TP3 n.s.
Significances, allergoid/MPL compared to allergoid alone, TP2 n.s., TP3 p=0.002.
Time points: TP1 = baseline, TP2 = before final injection, TP3 = 14 days after final injection, TP4 = 5 months after final injection.

Discussion

Several studies have shown that type 1 allergic patients elicit a distinct stronger T-cellular immune *in vitro* response to appropriate allergens than non-atopic patients [5,6,7]. In contrast, one study found no difference in the T-cell reactivity of atopics and non-atopics [8]. It is evident that the T-lymphocytes take part in the process of specific sensitisation since a successful hyposensitisation results not only in an improvement of the clinical symptoms, but also in a clear reduction of the late phase reaction following intracutaneous allergen application [9,1]. The SIT-induced modulation of the activity of T-helper cells leading to a changed lymphocyte cytokine profile is presumed to result from interaction of the active components of the vaccine (ie allergens or allergoids) with the cellular immune system.

The aim of this study was to examine whether (a) an *in vitro* specific proliferative T-cell response can be demonstrated by a LTT after stimulation with the exact vaccine preparations used in the SIT treatment of pollen-allergic patients (i.e. allergoids in this instance), and (b) how the lymphocyte activation varies over the course of a SIT. Our preliminary LTT experiments showed that the tyrosine-adsorbed allergoid vaccine (Pollinex® Quattro) was clearly able to induce a dose-dependent specific T-cell activation *in vitro*, and therefore this preparation qualified for use as a test antigen. This vaccine has a modern formulation, with the benefit of the Th1-inducing adjuvant MPL®, a detoxified lipopolysaccharide component of *Salmonella minnesota*. MPL® has also been employed as a useful Th1 adjuvant in anti-infective vaccines (e.g. malaria, hepatitis B, HSV-2 and Influenza). Control procedures with the LTT usefully showed that MPL® did not cause a non-specific reaction. The grass (and/or mugwort) pollen allergic patients without clinical or serologic signs of a tree pollen sensitisation showed a negative reaction to the tree pollen allergoid with MPL®, and a positive reaction to the grass/rye allergoid without MPL®. The exclusion of non-allergen specific activating effects through MPL® was particularly important, because MPL® has the dose-dependant property to induce the release of IFN-γ and IL-2 from lymphocytes or IL-1 from monocytes as well as inhibiting IL-4 production from Th2 cells [10,11]. The lymphocyte transformation test applied in this study is presumably stabilized by the addition of IFN-α. On the one hand IFN-α has antiproliferative effects by suppressing the proliferation of non-specific activated lymphocytes; on the other hand, it improves the antigen presentation of monocytes and the proliferation of specifically activated T cells [2]. These bivalent qualities of IFN-α could prevent non-specific activation *in vitro* due to the adjuvant.

In this respect it is interesting that MPL® also improves the function of the antigen-presenting cells [12]. This is probably why in our study, the mean stimulations were higher when using Pollinex Quattro preparations as stimulants compared to allergoid preparations without adjuvant.

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These preliminary experiments show that the LTT method is suitable for monitoring the specific *in vitro* T cell activation during a therapeutic trial of specific immunotherapy.

During this study, we investigated the *in vitro* proliferative response to allergoid preparations during a four-shot specific immunotherapy in 21 grass (and/or mugwort) pollen-allergic patients before the first injection, before the final injection and then 14 days and 5 months after the final injection. A significant increase of the *in vitro* specific immune response to allergoid stimulation was seen with highest values at the highpoint of therapy and a drop to baseline levels or below in the follow-up period.

The simultaneous *in vitro* tests using tree pollen allergoids excluded the possibility that the SIT with grass/rye (and/or mugwort) allergoids plus MPL® causes a global pre-activation of T-cells which influence the *in vitro* results. Furthermore, it was unlikely that the increase of stimulation indices in course of a treatment cycle are due to the *in-vitro* enhancing effect of the MPL® adjuvant.

As far as we know, these are the first published investigations of *in vitro* lymphocyte activation during a therapeutic trial with allergoids using the therapeutic preparations itself as *in vitro* stimulant. Furthermore, the *in vitro* allergoid-specific immune response was monitored in the initial phase of the therapy (after three injections) in contrast to other investigators who compared LTT results some months after the therapeutic trial of long injection courses. Nevertheless, these studies confirm earlier results that demonstrate that there is a late decrease in the allergen specific induced T-cell proliferation. SIT with grass pollen was shown to induce a distinct suppression of allergen specific lymphocyte proliferation [7]. A decrease was found in allergen-induced T-cell responses after a continuous SIT for three months [13]. The decrease of the T-cell proliferation showed in atopic patients undergoing grass pollen that SIT was not associated with a parallel drop in the specific IgE antibody levels [6]. However, the vaccine used in this study (Pollinex Quattro) did not induce an early IgE response and also prevented the seasonally-induced IgE boost that is normally seen [3]. This finding was probably due to the Th1-directing activity of MPL® adjuvant in the vaccine**.** The decrease of allergen-induced lymphocyte response was confirmed in pollen sensitive patients during nasally applied SIT [14].

The reduction of allergen-specific induced lymphocyte proliferation displayed in other studies could be due to a re-orientation of the T-helper cell reaction as a consequence of the SIT or possibly the result of the transfer of allergen-specific T-cells from the circulation into the interstitium. Nevertheless, it has been shown that specific immunotherapy leads to a reduction of the T-cell infiltration into the skin that does not support the idea of a simple transfer of cells [9].

We have not observed other reports where T-cell reactivity was elevated so early in an SIT injection course. Although other investigators may have missed this observation it is possible that the response could be partly due to the immunostimulating effect of MPL® in the vaccine formulation, associated with induction of IL-10 and IL-12.

We have shown that an allergen-specific T cell reaction in an early phase of a specific immunotherapy can prove the immunological response to allergen application. The clinical results showed an improvement in symptoms for all SIT patients, which was very encouraging from a treatment viewpoint. However, our results so far cannot predict whether the individual immunological response to the allergoids applied will always be associated with a clinical improvement of the symptoms. To examine whether the process is fully suitable for SIT monitoring it will be necessary to record well-detailed clinical parameters simultaneously in studies including a larger number of patients.

One must also consider that a decrease of allergen-induced proliferation also occurs during the pollen season [15]. Therefore, to correlate a therapeutic effect with a reduced allergen-specific stimulated lymphocyte proliferation it will be necessary to determine the T-cell activation pre-seasonally.

Until recently there was no effective and reliable *in vitro* method to investigate the cellular immunological effect of SIT. The examination of the specific stimulated lymphocyte proliferation after only a few injections could identify the patients whose PBMCs do or do not interact with the allergens or allergoids applied. The initial activation observed after only three injections reported in this study might provide a useful early way to distinguish good from poor clinical responders to allergy vaccination.

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