

Reactivity of T Cells with Grass Pollen Allergen Extract and Allergoid

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Key Words

Grass allergen · Grass allergoid · T cell · Peripheral blood mononuclear cells · Interleukin-5 · Interferon- γ · Antigen presentation · Dendritic cell · T cell clones · Phl p 5

Abstract

Background: Successful allergen-specific immunotherapy is achieved with progressively increasing doses of allergen or allergoid. In order to gain further insight into the mechanism of action of allergoids several in vitro investigations were conducted. **Methods:** Peripheral blood mononuclear cells (PBMC) from grass pollen allergic and nonallergic subjects were stimulated with either grass pollen extract or allergoid and the proliferation and cytokine production (IL-5, IFN- γ) were measured. Similar investigations were performed with Phl p 5-specific T cell lines (TCL) and clones (TCC). Dendritic cells and PBMC were compared in terms of their relative efficacies as antigen-presenting cells. **Results:** Both allergen and allergoid induced proliferation and Th2 and Th1 cytokine synthesis by PBMC of allergic subjects, whereas PBMC of nonallergic subjects did not produce IL-5. The maximum level of IL-5 was obtained with a lower concentration than was necessary for maximal IFN- γ production. Higher stimulation doses of allergen and allergoid shifted the cytokine profiles towards a Th1 phenotype. TCL and TCC clearly showed reactivity with both allergen and allergoid when using autologous

PBMC for antigen presentation, but compared with the native allergen the reactivity of the allergoid was reduced with most of the TCC. Using dendritic cells for antigen presentation a pronounced increase of stimulation of the TCC especially for the allergoids becomes obvious. **Conclusion:** In common with grass pollen allergen the corresponding allergoids possess a strong allergen-specific T cell-stimulating capacity. However, the degree of T cell stimulation by the allergoid seems to be dependent on the type of the antigen-presenting cell. Both, allergen and allergoid, can modulate T cell responses in a dose-dependent manner.

Introduction

The efficacy of specific immunotherapy with allergens and allergoids for the treatment of IgE-mediated allergic diseases has been verified by numerous clinical studies [1–4], but so far the underlying mechanism of the beneficial effect is not completely understood. Present knowledge suggests that there is a shift within the T cell subpopulation from a Th2 phenotype towards a Th1 phenotype during the course of immunotherapy [5–11].

Allergoids are chemically modified allergens. Earlier studies proved that although the allergenicity of allergoids is substantially reduced they retain their immunogenicity since they induce strong allergen-specific IgG antibody responses with comparable titers as with the allergens

[12–18]. It is well established that the synthesis of IgG and IgE are T cell-controlled processes and thus it was concluded that T cell epitopes must still be present after the chemical modification of allergens. One study has shown similar T cell-stimulating properties for house dust mite allergen and allergoid when used to stimulate peripheral blood mononuclear cells (PBMC) from house dust mite allergic and nonallergic subjects [18]. In another study effective allergoid therapy was associated with a significant reduction of interleukin-5 (IL-5) production in nasal secretions accompanied by increased IFN- γ production [8] suggesting the involvement of T cells in this beneficial effect.

In order to gain further insight into the mechanisms of action of allergens and allergoids, we conducted in vitro investigations to assess the influence of concentration on proliferation and production of Th1 and Th2 cytokines from human T cells. For this purpose PBMC from grass pollen-allergic subjects and nonallergic donors were used for investigations on the polyclonal level. Furthermore, T cell lines (TCL) and clones (TCC) were established from grass pollen-allergic subjects following stimulation with the major allergen of *Phleum pratense*, Phl p 5, and were then used for similar stimulation experiments. As is usual in proliferation studies we used autologous PBMC for antigen presentation. However, PBMC are not the principal target antigen-presenting cells (APC) during the course of specific immunotherapy. In nonlymphoid tissues the most potent APC are dendritic cells (DC) which are essentially important for the initiation of primary T cell-mediated immune responses to foreign antigens in the direction of the Th1 phenotype [19]. Several methods have recently been described for the generation of DC from progenitors in human blood under cultivation with GM-CSF and IL-4 [20–24], and we took advantage of this to extend our investigation into the use of this type of APC.

Materials and Methods

Reagents

A commercially produced grass pollen extract (Allergopharma, Reinbek, Germany) derived from pollen of *P. pratense*, *Lolium perenne*, *Holcus lanatus*, *Dactylis glomerata*, *Poa pratensis* and *Festuca pratensis* (Allergon, Engelholm, Sweden) was used. The production procedure involved extraction in Coca's solution (5 g NaCl and 2.5 g NaHCO₃/l) at 4°C for 16 h, centrifugation, diafiltration (molecular weight cutoff 5 kD) and lyophilization. Allergoids were produced from characterized and standardized extracts by treatment with formaldehyde according to Marsh [14].

Purified major allergen Phl p 5 was prepared by affinity chromatography using the monoclonal antibody 1D11 [25, 26] coupled to protein G Sepharose 4 fast flow (Pharmacia, Freiburg, Germany) by a method described elsewhere [27].

Blood Donors

PBMC for direct stimulation experiments and generation of TCL were obtained from grass pollen allergic subjects with a clinical history of hay fever and Enzyme Allergo Sorbent Test (EAST) classes ≥ 3 (specific IgE ELISA – RV, Allergopharma) with grass pollen or *Phleum pratense* pollen extract. PBMC from subjects with no allergy and no grass pollen-specific IgE were used for controls.

Isolation of PBMC

Isolation of PBMC from the blood of allergic and nonallergic donors was performed with Lymphoprep media (density = 1.077; Life Technologies, Eggenstein, Germany) in Leucosep tubes (Greiner, Frickenhausen, Germany) according to the instructions of the manufacturers.

Stimulation of PBMC

All stimulation experiments were performed in serum-free Ultraculture medium (Bioproducts, Heidelberg, Germany) supplemented with 2 mM Glutamax I (Life Technologies), antibiotic-antimycotic solution (Sigma, Deisenhofen, Germany) and 20 μ M β -mercaptoethanol (Life Technologies). The stimulations were performed with different concentrations of grass pollen extract or allergoid manufactured from the same batch of extract. The allergen and allergoid were adjusted to different concentrations in terms of protein nitrogen units (PNU)/ml according to Stull et al. [28] and Taylor and Francis [29]. PNU values were chosen because this parameter allows a direct comparison of allergen and allergoid concentrations independent of the respective condition. 100 PNU correspond to approximately 6.25 μ g protein [29, 30].

The stimulation was set up with 2×10^5 PBMC per well in 96-well round-bottom culture plates and allergen concentrations ranging from 0.01 to 2,500 PNU/ml in a total volume of 200 μ l/well. Each allergen concentration was tested in 4–6 replicates. 100 μ l of culture supernatant was removed from each well after 6 days, supernatants obtained from the replicates were pooled, centrifuged and stored at -20°C until assayed for cytokines. The cultures were supplemented with 75 μ l medium. Subsequently 25 μ l containing 1 μ Ci of ^3H -thymidine (Amersham, Braunschweig, Germany) was added and the cultures incubated for a further 16 h. Cells were harvested as described under 'Proliferation Assay'.

Generation of Allergen-Specific TCL

Phl p 5-specific TCL were obtained as described by Müller et al. [31] with several modifications: 1×10^5 PBMC and 10 μ g Phl p 5/ml Ultraculture were seeded in a 96-well round-bottom culture plate in a total volume of 100 μ l/well. After 6 days the cultures were fed by adding 100 μ l Ultraculture supplemented with 25 U IL-2/ml (PBH, Hannover, Germany). 3–4 days later half of the culture medium was exchanged for fresh medium containing 25 U IL-2/ml and incubated for another 4 days. 14 days after establishing the cultures a proliferation assay was performed by dividing the growing T cells between three 96-well plates. One plate was kept in culture for expansion of the desired T cells, the other two plates were subjected to the proliferation assay, one by incubation with the allergen the other without allergen. For a TCL the cells from 12 to 16 culture wells with stimulation index (SI) ≥ 3 were pooled. Up to three different TCL were collected from one 96-well plate depending on the stimulation indices for the individual. The TCL were cloned immediately and expanded as described below.

Generation of Allergen-Specific TCC

Selected Phl p 5-specific TCL were cloned by seeding 0.3 T cells/20 μ l Ultraculture medium under stimulation with 5 μ g Phl p 5/ml, 50 U IL-2/ml, 5 U IL-4/ml and 1×10^4 irradiated autologous PBMC per well in Terasaki culture plates (Greiner). The plates were checked for growing clones after 7–10 days. Growing clones were expanded in 96-well culture plates under stimulation with 5×10^4 irradiated autologous PBMC per well, allergen and cytokines in the same concentrations as described above. The clones were cultured by changing half of the medium every 3–4 days with fresh medium supplemented with 25 U IL-2/ml. 14 days after feeding with autologous PBMC cells were assayed for allergen-induced proliferation response.

Expansion of TCL and TCC

TCL and TCC were expanded by stimulation with Phl p 5 (5–10 μ g/ml) and $1\text{--}2 \times 10^6$ autologous PBMC irradiated at 30 Gy in a 24-well culture plate. After 3–4 days the cultures received an unspecific stimulation for optimal growth using 2.5 μ g phytohemagglutinin (Seromed/Biochrom, Berlin, Germany)/ml Ultraculture, 25 U IL-2/ml and $1\text{--}2 \times 10^6$ allogenic PBMC (a mixture of PBMC from at least 5 nonallergic donors) irradiated at 30 Gy. Cells were expanded by exchange of half of the medium supplemented with 25–50 U IL-2/ml every 3–4 days. Expanded and tested TCL and TCC were stored in liquid nitrogen. A proliferation assay was set up at least 14 days after the last PBMC incubation. For longer culture periods cells received an allergen stimulation with autologous irradiated PBMC every 4 weeks.

Proliferation Assay

For the proliferation assay cells of TCL or TCC were seeded at 2×10^4 cells/well of 96-well culture plates in triplicate under stimulation with the allergen (5–10 μ g Phl p 5) and 5×10^4 irradiated (30 Gy) autologous PBMC. After 48 h in the incubator 1 μ Ci ^{3} H-thymidine was added to each well and the plates were further incubated for 16 h. Cells were then harvested on Microbeta filter mats with a 96-well cell harvester (Wallac ADL, Freiburg, Germany). The filter mats were dried and the solid scintillator Meltilex was melted into these filter mats using a microplate heat sealer (Wallac ADL). Filter mats prepared in this way were transferred in filter cassettes and counted in a Microbeta scintillation counter (Wallac ADL). The SI was calculated as the quotient of counts per minute (cpm) of the allergen stimulation (T cells, irradiated autologous PBMC and allergen) and the unstimulated control (T cells and irradiated autologous PBMC). In some assays DC were used instead of autologous PBMC in a concentration of 5×10^3 DC/well.

Generation of DC

DC were cultivated from CD14⁺ precursors of autologous PBMC according to the methods described in the literature [20–23] with slight modifications: CD14⁺ cells were obtained by labeling the PBMC with anti-CD14 monoclonal antibody-coupled paramagnetic microbeads and subsequent separation of the desired cells on a column placed in a MiniMACS separator (Miltenyi Biotec, Bergisch Gladbach, Germany). CD14⁺ cells were cultivated in Ultraculture medium (BioWhittaker, Verviers, Belgium) supplemented with 2 mM Glutamax I (Life Technologies), antibiotic-antimycotic solution (Sigma) and 20 μ M mercaptoethanol (Life Technologies) and addition of 200 ng IL-4 (Biosource, Ratingen, Germany) and 60 ng GM-CSF (Biosource)/ml. 5×10^6 CD14⁺ cells were seeded in a total volume of 1 ml in 24-well culture plates (Nunc, Wiesbaden, Germany). Every

second or third day half of the medium was removed and substituted with fresh medium containing both cytokines in equal amounts as described above. After 7 days of culture the cells were harvested and used as APC for autologous TCC in proliferation assays. Cells grown under these conditions from CD14⁺ precursor cells are considered as immature DC with a strong antigen capturing and processing capacity [23, 32]. The cells correspond to the phenotype of immature DC (CD14⁺/low, CD16⁺, CD64⁺, CD83⁺, CD86^{high}, HLA-DR⁺) as evidenced by FACS analysis and microscopic control (unpubl. data).

Cytokine Measurement

The cytokines IL-4, IL-5 and IFN- γ were analyzed using Cytoscreen ELISA kits (Biosource) according to the instructions of the manufacturer in culture supernatants of T cells and PBMC. The supernatants were obtained after 48 h incubation periods for TCL and TCC and after a 6-day culture period for PBMC.

Statistics

For statistical evaluation the Mann-Whitney rank sum test (Software: Jandel Sigma Stat, Version 2.0) was used to compare the cytokine production as well as the cytokine ratio of PBMC from allergic and nonallergic subjects of cultures with the same concentrations of allergen and allergoid. Probability values of $p < 0.05$ were considered significant. The Wilcoxon signed rank test was used to compare the values of different concentrations for stimulation within a respective group and for the evaluation of possible differences in the cytokine ratio between stimulation of allergen as well as allergoid within a group.

Results

Allergen as well as Allergoid Induce Proliferation of PBMC from Allergic or Nonallergic Donors

Cells from grass pollen-allergic donors as well as donors without clinical symptoms of allergy were used to investigate the proliferation of PBMC in response to grass pollen extract and allergoid. The proliferation of PBMC after stimulation with the grass pollen allergen as well as the allergoid was dependent on the antigen dose and was maximal after stimulation with a high protein concentration of 2,500 PNU/ml. There was no difference when comparing the proliferation between allergen and allergoid in both groups (fig. 1a, 2a). PBMC from nonallergic donors showed a similar proliferation profile as the allergic donors with both stimulants, the allergen and allergoid.

Background counts and maximal levels between the individual subjects varied considerably and therefore results were expressed as an SI. The background values for the allergic subjects were 3,500–14,500 cpm; maximal values obtained with the grass pollen extract were 13,400–100,000 cpm, and with the allergoid 13,600–92,500 cpm. In the case of the nonallergic subjects the background values were between 2,900 and 12,000 cpm, between 20,700 and 64,700 cpm with the extract and between 17,500 and 92,500 cpm

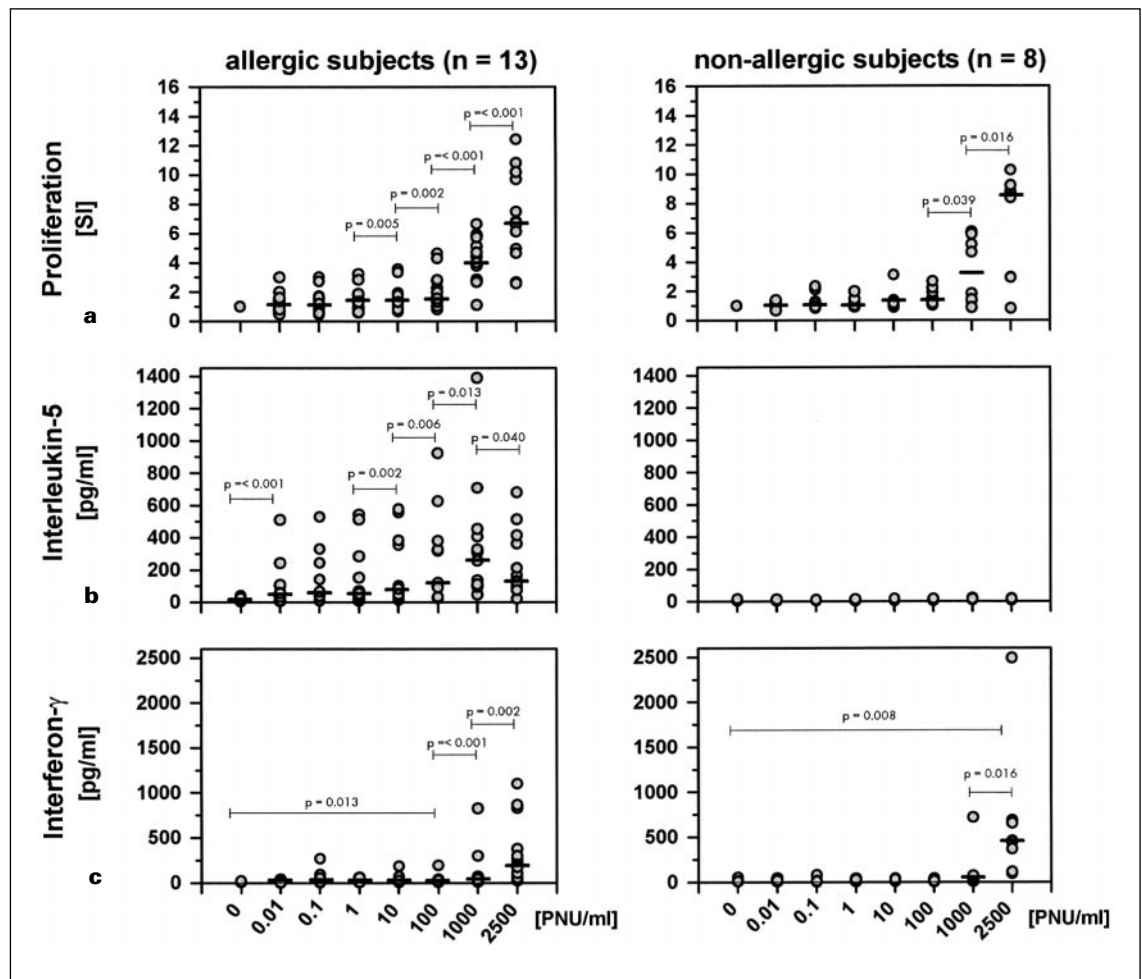


Fig. 1. Proliferation and synthesis of the cytokines IFN- γ and IL-5 from PBMC of grass pollen allergic and nonallergic subjects after stimulation with different concentrations of grass pollen allergen. The horizontal bars mark the median values. Values for p were obtained with the Wilcoxon signed rank test.

with the allergoid. It was individually different if the highest proliferation response was obtained with the extract or the allergoid and sometimes the differences were considerable.

Allergen as well as Allergoid Induce Different Cytokine Profiles in PBMC of Allergic or Nonallergic Donors

After stimulation with different concentrations of allergen (fig. 1b, c) or allergoid (fig. 2b, c) the culture supernatants of PBMC from allergic and nonallergic donors were tested for IL-5 and IFN- γ synthesis. IFN- γ was synthesized after stimulation with higher doses of the native allergen extract both in allergic and nonallergic donors, with the latter producing higher average amounts of this cytokine, al-

though this difference was not statistically significant (fig. 1c).

In contrast to IFN- γ production, IL-5 was produced in statistically significant amounts ($p < 0.05$) even with very low protein doses as compared with the control stimulation (fig. 1b). The maximal production of IL-5 was obtained with lower protein concentrations (1,000 PNU/ml) as compared with IFN- γ (2,500 PNU/ml; fig. 1b, c). In contrast to the allergics, the IL-5 synthesis in nonallergic donors was at a very low level (fig. 1b).

When looking at the allergoid stimulations only high concentrations led to IFN- γ production (fig. 2c). The IL-5 production showed a similar course as that with the extract stimulations (fig. 2b) and the low levels obtained with the

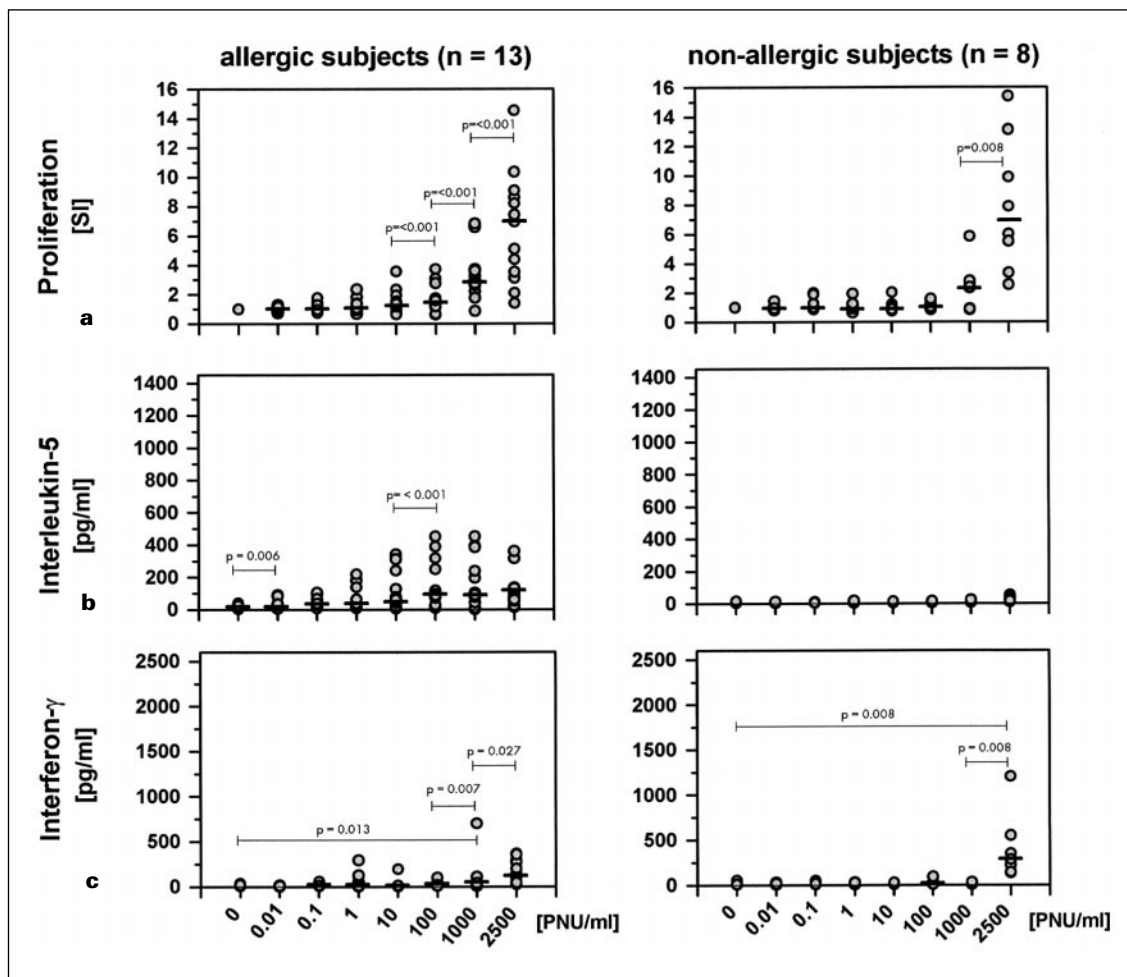


Fig. 2. Proliferation and synthesis of the cytokines IFN- γ and IL-5 from PBMC of grass pollen allergic and nonallergic subjects after stimulation with different concentrations of grass pollen allergoid. The horizontal bars mark the median values. Values for p were obtained with the Wilcoxon signed rank test.

allergoid were not statistically significant. Again in nonallergic donors IL-5 production was very low. When comparing the IL-5 production of allergic with nonallergic subjects, all stimulations, including the control, yielded significantly higher values in the allergic group with both the allergen and the allergoid ($p < 0.01$).

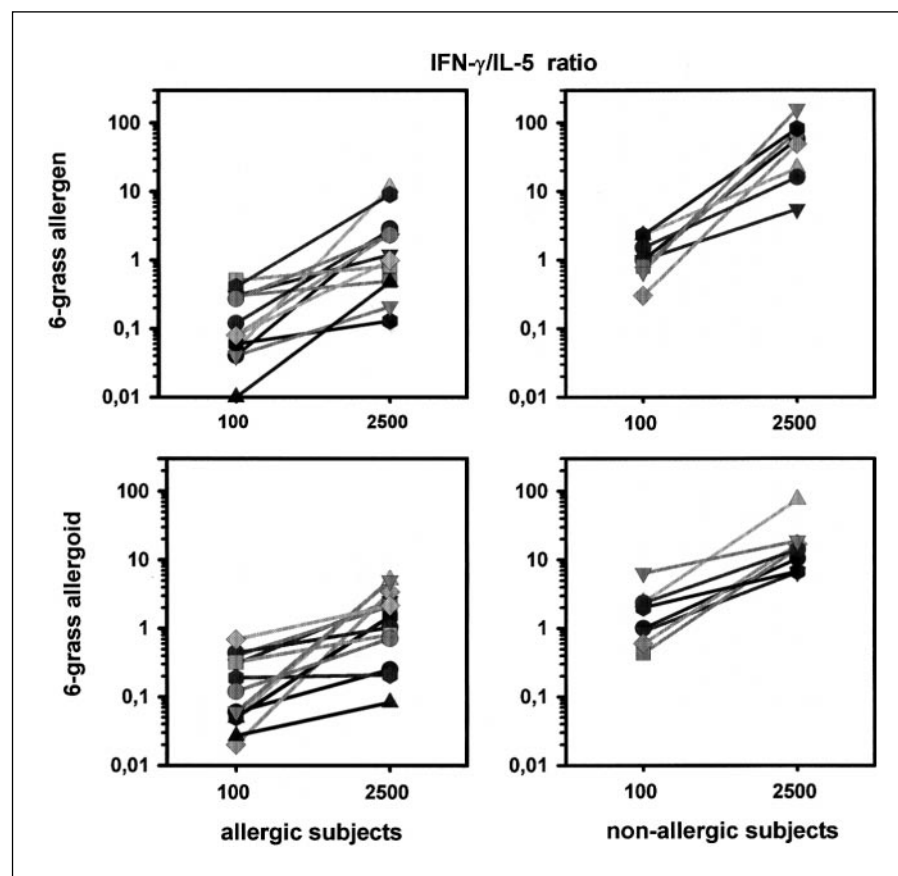
High Concentrations of Allergen as well as Allergoid Favor a Shift towards a Th1 Cytokine Profile

Since IFN- γ is considered as a typical Th1 cytokine and IL-5 a Th2 cytokine, the ratio between the two might be a parameter for characterizing the immune response in vitro. We calculated the IFN- γ /IL-5 ratio by using the highest (2,500 PNU/ml) and lowest effective doses (100 PNU/ml)

for both stimulants for most of the blood donors (fig. 3). Stimulation of PBMC from allergic donors with the native allergen extract showed a Th2 response with the low doses (100 PNU/ml). Using the higher allergen extract concentration a clear shift towards a Th0/1 response was evident. The nonallergic group showed a Th0 cytokine response with the low stimulation dose and a clear Th1 phenotype with the higher allergen dose. The IFN- γ /IL-5 ratio was significantly higher ($p < 0.01$) in nonallergic individuals as compared with allergic subjects with both stimulation doses of allergen and allergoid.

When comparing the IFN- γ /IL-5 ratios following allergen and allergoid stimulation in the allergic group, the shifts towards the Th1 cytokine profile were comparable (table 1).

Fig. 3. IFN- γ /IL-5 ratio after stimulation of PBMC from allergic and nonallergic subjects with low and high doses of grass pollen allergen and allergoid. Identical symbols within the graphs of the allergic or nonallergic subjects represent the values obtained from the same individual.



The ratios for the 100 and 2,500 PNU/ml stimulations between allergen or allergoid are not significant within the allergic and nonallergic group. The magnitude of the shift towards Th1 with the allergen or allergoid varied for the individual patients, some patients showing a stronger shift with the allergen, other patients with the allergoid (fig. 3).

Grass Pollen Allergoids Retain Stimulating Activity for TCL and TCC

The proliferative responses of several of the Phl p 5-specific TCL were compared after stimulation with the grass pollen extract and allergoid. The TCL showed different thresholds of activation also in respect of reactivity towards the allergen extract and allergoid (fig. 4). The spectrum of reactivity of the TCL ranged from similar for the allergoid and unmodified allergen, to more or less reduced reactivity. When looking for the cytokine production of TCL after stimulation with allergen and allergoid, in most cases allergoid stimulation resulted in reduced production of both Th2 and Th1 cytokines (data not shown).

In addition to the investigations with TCL the reactivity of Phl p 5-specific TCC from different patients were also

Table 1. IFN- γ /IL-5 ratio of PBMC derived from allergic and nonallergic donors after stimulation with low or high doses of grass pollen allergen or allergoid

Stimulation with	Allergic donors		Nonallergic donors	
	100 PNU/ml	2,500 PNU/ml	100 PNU/ml	2,500 PNU/ml
Allergen	0.08	1.20	1.00	53.15
Allergoid	0.12	1.50	1.50	14.23
p value	= 0.831	= 0.588	= 0.578	= 0.109

The values in the table represent the medians; the statistical evaluation was performed with the Wilcoxon signed rank test.

compared. Dose-response reactivity with several of the TCC revealed concentrations of 100 PNU/ml of grass pollen extract or allergoid as optimal for the majority of stimulations (fig. 5). In most cases higher protein concentrations showed a suppressive effect on the proliferation of both TCL and TCC. Taking the 100 PNU/ml stimulation value we evaluated the allergoid reactivity from a panel of

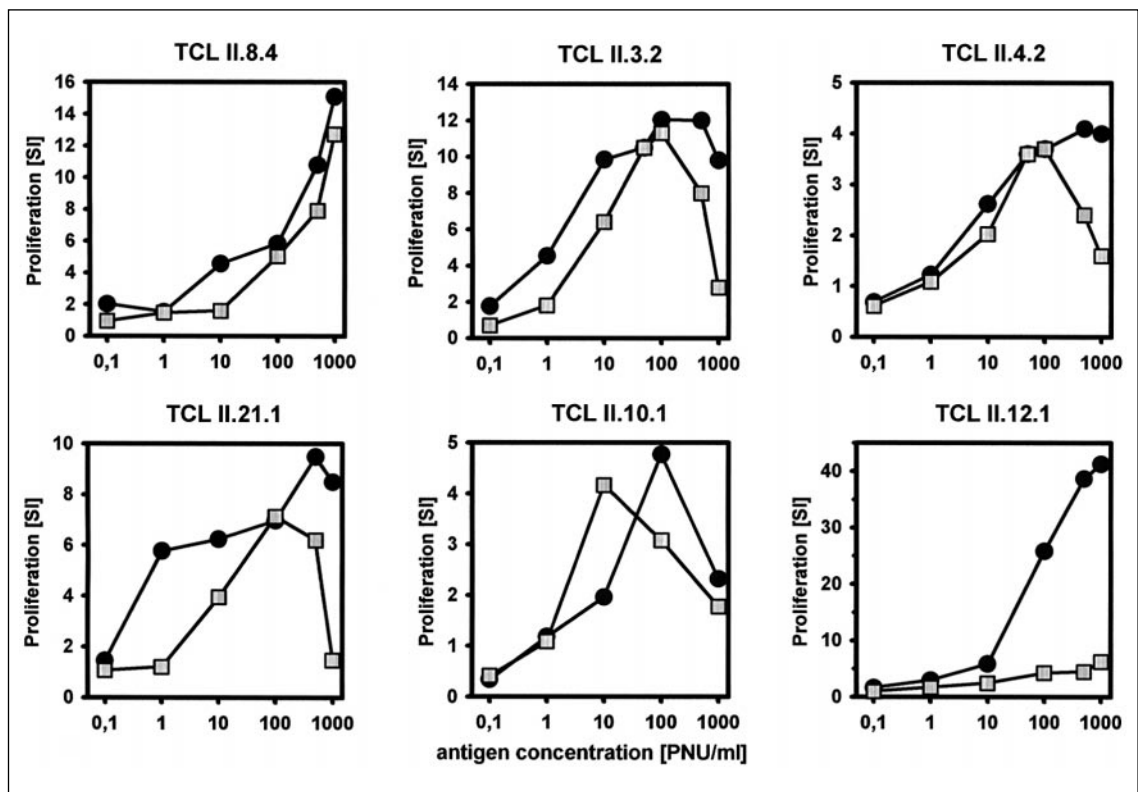


Fig. 4. Proliferation patterns of Phl p 5-specific TCL from different grass pollen-allergic subjects after stimulation with different concentrations of grass pollen allergen (●) or allergoid (□). Concentrations are expressed in PNU/ml of grass pollen allergen and allergoid.

33 TCC from 8 different patients which gave a reactivity of $SI \geq 3$ with Phl p 5 (10 $\mu\text{g/ml}$) as well as grass pollen extract (100 PNU/ml; table 2). The majority of the TCC (76%) showed an $SI \geq 3$ with the allergoid. In most cases the reactivity of T cells to allergoid was lower than to the native allergen to various degrees when using the autologous PBMC for antigen presentation. Much higher proliferative responses were obtained for both stimulants when using DC for presentation (fig. 6). Even TCC with weak proliferation in response to allergoid presented by PBMC showed a much stronger proliferation when antigen presentation was performed by DC. With these specialized APC the grass pollen allergoid proved to be as powerful as the native allergen for T cell stimulation. The relative reactivity in response to allergen and allergoid varied between the individual clones but stimulation by the allergoid was always higher when presentation was performed by DC as opposed to PBMC.

The importance of this type of APC for the presentation of allergoids was most obvious for TCC, several of which did not fulfill the criteria to be included in table 2 because in combination with PBMC as APC they did not achieve an

$SI \geq 3$ with the grass pollen allergen although with their antigen Phl p 5, which was used for their selection, they yield considerable proliferation. Even these clones show a strong proliferation with both components when presented by DC (e.g. clone II.16.1: 27A3 in fig. 6). The absence of the relevant allergen in cultures of Phl p 5-specific TCC and autologous DC did not lead to any stimulation of the TCC.

Discussion

One advantage in the use of allergoids for specific immunotherapy is their greatly reduced IgE reactivity as compared with the native allergen which allows a more rapid progression to higher concentrations and thus fewer injections and less IgE-mediated side effects during therapy. In addition to the results of earlier studies which demonstrated a strong reduction of IgE binding to allergoids as compared with the natural allergen extract [13, 14], we are now able to demonstrate that allergoids retain pronounced T cell reactivity.

After stimulation of PBMC with both components under investigation we found no difference in the proliferative response between allergic and nonallergic subjects and between allergen and allergoid. Since the nonallergic subjects possess strong IgG binding with various components of the grass pollen extract (data not shown) it is not surprising that immune-competent cells are stimulated in this polyclonal system. The observation that nonallergic subjects show a proliferation in response to allergens is in accordance with other authors although there is some controversy as to whether the proliferation of PBMC from allergic and nonallergic subjects is similar or lower for the latter [33–38].

When looking for the cytokine production of PBMC after stimulation both allergic and nonallergic subjects show different cytokine profiles. We chose to measure IL-5 as a typical Th2 cytokine because it was detectable in reasonable quantities in PBMC culture supernatants, whereas the often used IL-4 was detectable only in extremely small amounts, often under the detection limit of the ultrasensitive ELISA systems (0.4–25 pg/ml; data not shown). Similar observations were made by others [38–42]. The reason may be a strong consumption of IL-4 by certain cells in the PBMC preparation.

The Th2 cytokine IL-5 was produced in substantial amounts only after stimulation of PBMC from allergic donors whereas nonallergic donors did not show any significant IL-5 production. Maximum IL-5 production was reached with lower stimulation doses as compared with the release of IFN- γ and pronounced IL-5 production is visible even with very low stimulation doses within the allergic group. The production of Th2 cytokines from PBMC of allergic subjects in contrast to nonallergic subjects has also been demonstrated by others for IL-5 [39, 41, 43] and for IL-4 with the PCR method [44].

The typical Th1 cytokine IFN- γ is produced in large amounts with increasing stimulation doses in PBMC from both allergic and nonallergic subjects, but the average IFN- γ production is higher from PBMC of nonallergic subjects.

Comparison of the cytokine production in response to allergen and allergoid shows that IFN- γ and IL-5 are produced in lower concentrations after stimulation with the allergoid. However, increasing concentrations of both preparations induced a pronounced shift towards a Th1 response as reflected by the IFN- γ /IL-5 ratio following stimulation of PBMC from allergic donors. But in contrast to native allergens the reduced allergenic activity of allergoids makes it possible to administer high doses early during the course of specific immunotherapy and so to reach the therapeutic effect more rapidly and without IgE-mediated side effects. The observation that Th1 and Th2 cytokine responses

Table 2. Reactivities of Phl p 5-specific TCC together with PBMC as APC after stimulation with their antigen Phl p 5 (10 μ g/ml) in comparison with grass pollen extract and grass pollen allergoid (100 PNU/ml)

Patient	TCC	SI _{Phl p 5}	SI _{6-grass extract}	SI _{6-grass allergoid}
3	2A3	4.2	11.0	5.1
	2A7	5.2	3.1	2.4
	2A12	29.0	12.0	3.3
	3D2	14.7	17.3	8.6
	4B8	21.9	65.6	58.3
	4E7	8.1	5.1	3.7
	5D6	30.0	25.9	15.0
	6H4	20.5	14.7	14.6
10	6G12	35.9	43.7	26.3
	1D6	9.0	4.6	3.6
12	21E7	11.5	3.7	1.3
	1C2	8.2	6.5	4.7
	2G7	4.3	6.1	5.1
	4C11	4.4	4.3	2.0
	4F3	3.6	3.8	2.8
	4G5	3.4	4.0	3.3
	5E2	3.1	3.4	2.5
	6A6	4.1	5.5	4.1
	7C8	14.5	19.9	8.0
	7F10	13.5	8.9	3.0
	7H7	4.4	3.0	3.1
	9C12	20.2	22.9	14.1
	9G9	4.2	4.2	2.0
	9H11	24.8	20.6	10.7
15	27C8	3.6	5.3	3.9
	29E7	17.5	22.8	12.0
17	1D8	11.1	8.8	6.4
	11C2	11.4	5.4	2.5
	19A1	11.6	7.5	5.1
19	6F7	9.1	5.2	3.7
	10C6	8.4	5.3	3.1
	21D9	8.6	5.2	3.0
20	9B6	3.8	4.9	2.6

Results are expressed as SI. Only those TCC which gave an SI of >3 with Phl p 5 and grass pollen extract are included.

es are favored by high and low allergen concentrations, respectively, is in accordance with the findings of other authors [45, 46]. Nonallergic donors show a clear tendency towards a Th1 response even with the lower stimulation doses. A higher IFN- γ /IL-5 ratio for nonallergic compared to allergic subjects has also been reported for PBMC stimulations with birch pollen allergen [43] by using a different stimulation protocol including allergen and PHA stimulations.

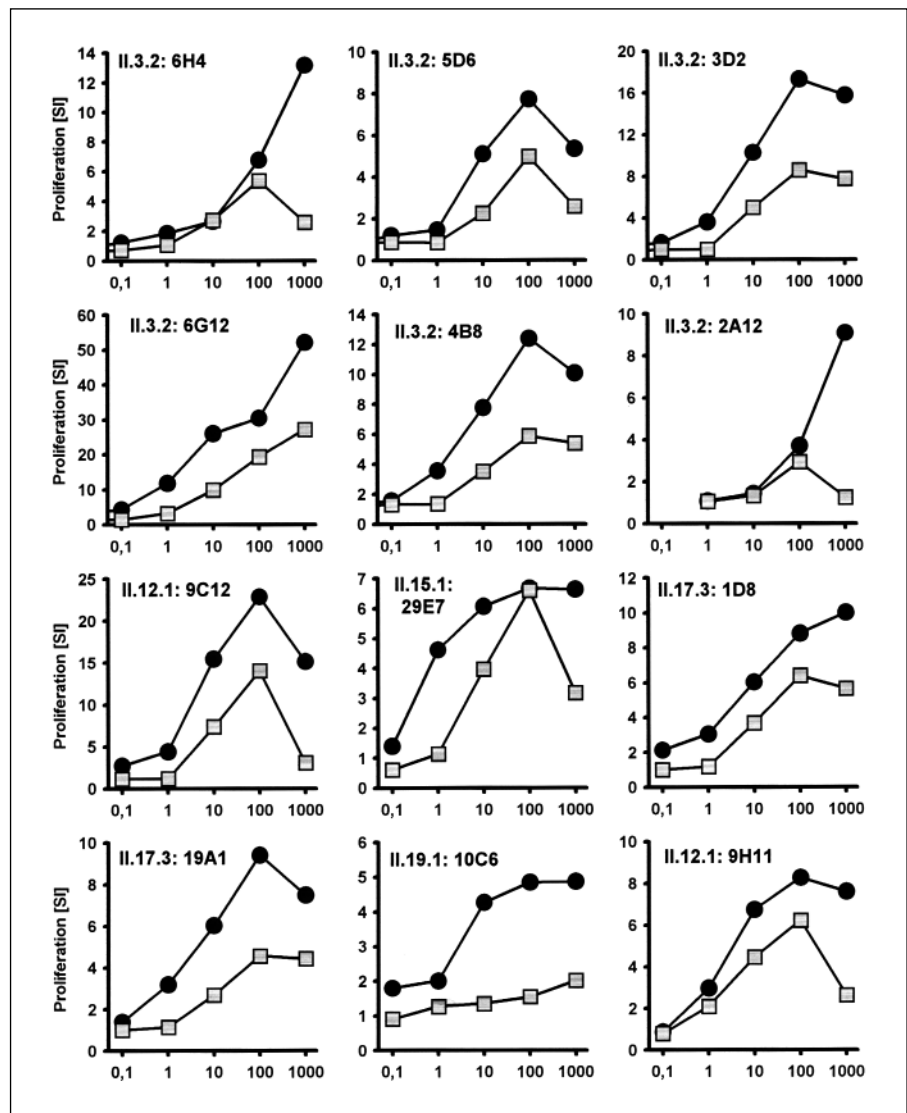


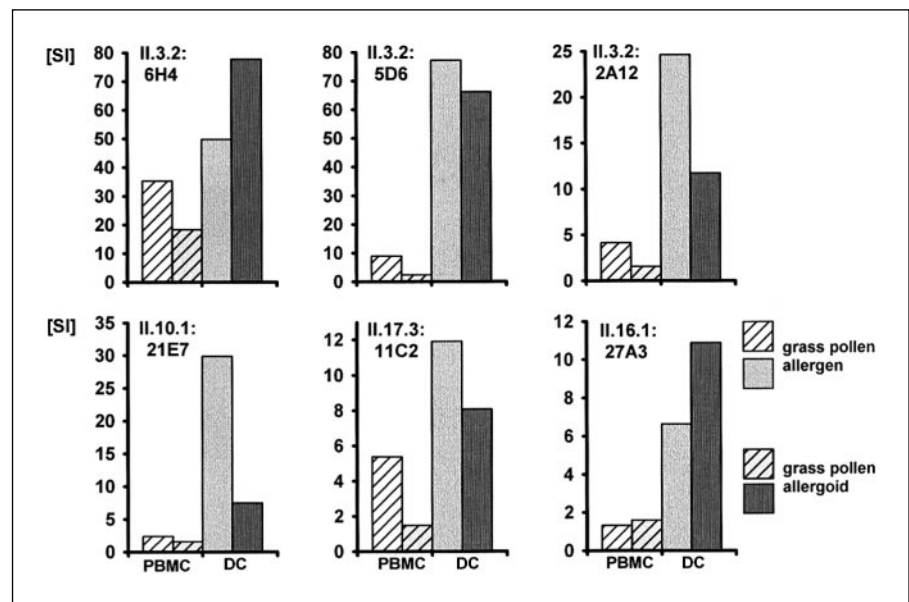
Fig. 5. Proliferation patterns of Phl p 5-specific TCC from different grass pollen-allergic subjects after stimulation with different concentrations of grass pollen allergen (●) or allergoid (□). Concentrations are expressed in PNU/ml of grass pollen allergen and allergoid.

As is usually the case in proliferation assays with Th cells antigen presentation was performed with autologous PBMC. We considered the possibility that these cells might contribute to the observed differences of the proliferative response between the native allergen and allergoid. It has been reported that antigen presentation via allergen-specific IgE bound to Fc ϵ RI is involved in T cell stimulation [47–50]. Since the PBMC in our study were obtained from allergic subjects, allergen uptake via IgE/Fc ϵ RI is highly probable. Furthermore allergen-specific B cells might operate as APC as well [51]. Akdis et al. [52] showed that memory B cells specific for the major bee venom allergen PLA2 were able to bind recombinant PLA2, refolded to the native conformation, with great efficiency leading to a Th2 cy-

tokine-dominated response. The nonrefolded PLA2, which in terms of IgE binding is comparable to an allergoid, was not bound and thus not presented by B cells. In contrast to B cells, monocytes bound, processed and presented the refolded as well as the nonrefolded PLA 2 and directed a Th1 immune response.

Under the conditions of specific immunotherapy performed by subcutaneous injection, DC and macrophages in the tissue rather than cells in blood are the first target for allergen presentation. For this reason we generated DC from CD14 $^{+}$ precursors in blood and compared these with autologous PBMC for presentation of allergen and allergoid to TCC. DC enhanced the proliferation responses of all TCC, demonstrating that allergoids, like allergens, can act as

Fig. 6. Comparison of the proliferative response (SI) of some TCC with grass pollen allergen and allergoid obtained with different populations of APC: autologous PBMC (5×10^4 PBMC and 2×10^4 T cells per well) or DC (5×10^3 DC and 2×10^4 T cells per well).



powerful allergen-specific T cell-stimulating substances. Since the outcome of T cell stimulation by allergoid as compared with the native allergen is different between PBMC can in vitro generated DC it is very probably that different mechanisms of antigen uptake and processing are involved. It seems likely that IgE/Fc ϵ RI-mediated mechanisms are involved or dominate the antigen uptake and presentation by PBMC from allergic subjects, and thus allergoids with their strongly reduced IgE binding have a clear disadvantage. In contrast DC can take up antigen very effectively by pinocytosis [32, 51, 53, 54]. Obviously antigen uptake by this non-specific mechanism accommodates both allergen and allergoid, and allergoids may be at an advantage due to their denatured native conformation. This can be concluded from the observation that the antigen presentation of allergen as well as allergoid by DC led to a pronounced stimulation of the TCC. Clones which were stimulated only weakly by the allergoid when using PBMC for presentation responded with strong stimulation upon presentation by DC.

Our results clearly demonstrate that allergoids as well as allergens can stimulate TCC and that differences are attributable to antigen uptake and presentation. However, in some cases there are quantitative differences in the stimulating capacity of allergen and allergoid. One possible reason for these differences may be that the formaldehyde-modified amino acid side chains (mainly ϵ -amino groups of lysine) may influence the binding of the allergoid-derived T cell peptide to the MHC II molecule and/or T cell receptor. It has been shown in several model systems that substitution

of single amino acid residues of T cell ligands can alter the affinity to the T cell receptor and/or MHC II molecules and thus T cell responses (proliferation, cytokine spectrum and quantity) [55–60]. Thus the observed differences in the degree of proliferation with PBMC as well as DC could be attributable to formaldehyde-modified T cell peptides. Reduced T cell proliferation could be found for example with TCC II.17.3: 11C2, II.3.2: 2A12 and II.10.3: 21E7 (fig. 6) whilst on the other hand, TCC II.16.1: 27A3 was stimulated more strongly by the allergoid-derived ligand, even with PBMC. All these TCC are specific for T cell epitopes which contain formaldehyde-reactive lysine residues in different positions [61].

The inability of allergoids to react with allergen-specific IgE antibodies and thus activate allergic effector cells such as basophils and mast cells via Fc ϵ RI avoids production of IL-4 and prevents upregulation of the allergic response [62–64]. Furthermore, the stimulation of the already elevated allergen-specific Th2 cells in allergic subjects by allergen presentation via B cells would not work for the allergoid, thus preventing further IL-4 production and a Th2-supporting milieu. During the course of specific immunotherapy newly stimulated T cells will be directed towards the Th1 phenotype.

Recent developments in the field of genetically modified allergens, which use the principle of allergoids with reduced IgE reactivity but retained T cell reactivity, might lead to more defined products for specific immunotherapy [61, 65].

In conclusion, allergoids combine the ability to activate T cells with reduced IgE reactivity, which in comparison with the native allergen confers distinct advantages for use in allergen-specific immunotherapy.

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