

Analysis of the Antibody Responses Induced by Subcutaneous Injection Immunotherapy with Birch and Fagales Pollen Extracts Adsorbed onto Aluminum Hydroxide

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Key Words

Epitopes · Immunotherapy · Peptides · Recombinant allergens

Abstract

Background: Allergen-specific subcutaneous immunotherapy (SCIT) is an antigen-specific therapy of IgE-mediated allergies. In the present study, we analyze the epitope specificities of antibody responses induced by SCIT with allergen extracts from pollen of trees belonging to the order *Fagales* (birch, alder, hazel) adsorbed onto aluminum hydroxide.

Methods: The IgE, IgG1–4 and IgA responses to defined recombinant allergens (birch pollen: Bet v 1; alder pollen: Aln g 1; hazel pollen: Cor a 1; apple: Mal d 1) as well as to Bet v 1-derived recombinant fragments and synthetic peptides were analyzed in sera from patients who had undergone SCIT for different periods of time. **Results:** Long-term SCIT (>1 year; cumulative dose >1,000,000 SQ units) induced more pronounced IgG1, IgG2 and IgG4 responses to Bet v 1 and Bet v 1-related allergens according to the degree of sequence homology (Bet v 1>Aln g 1>Cor a 1>Mal d 1) than short-term SCIT (<1 year; cumulative dose <1,000,000 SQ

units). In contrast to patients treated for <1 year, patients treated for >1 year mounted distinct IgG1, IgG2 and IgG4 responses against sequential Bet v 1 epitopes. No relevant allergen-specific IgA or IgG3 responses were induced by short- or long-term SCIT. Using a competitive ELISA assay, it could be shown that serum IgG from patients undergoing long-term SCIT inhibited IgE reactivity to Bet v 1 better than IgG from patients undergoing short-term SCIT. **Conclusion:** SCIT with allergen extracts adsorbed onto aluminum hydroxide induces IgG responses against new epitopes that block IgE binding and cross-react with structurally related allergens depending, among other factors, on duration of treatment and cumulative injected dose.

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Introduction

Pollen from trees of the order *Fagales* (e.g. birch, alder, hazel, hornbeam and oak) belong to the most important allergen sources in northern and central Europe as well as in certain areas of North America [1]. The allergens of these trees have been characterized in great detail down

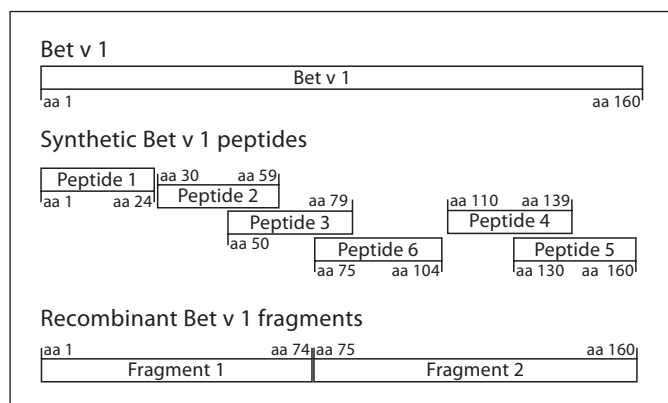


Fig. 1. Schematic illustration of the complete recombinant Bet v 1 protein, of the 6 synthetic linear Bet v 1-derived peptides and the two recombinant Bet v 1 fragments. aa = Amino acid.

to the molecular and structural level [1]. Although *Fagales* pollens contain several allergens, all patients sensitized to *Fagales* pollen show IgE reactivity and clinical sensitivity to the major allergen of birch, Bet v 1, and more than 65% of the patients are exclusively sensitized to this allergen [2, 3]. IgE and T cell cross-reactivity with Bet v 1-related allergens in various plant foods (fruits, vegetables and spices) is responsible for symptoms of food allergy (i.e., oral allergy syndrome) in patients allergic to *Fagales* pollen [4, 5].

The most established form of treatment against *Fagales* pollen sensitization nowadays is subcutaneous immunotherapy (SCIT) with birch pollen extract or treatment with *Fagales* pollen extract. Both forms of therapy were shown to be equally effective in an extensive analysis published in 1988 [6–8]. However, a study published 2 years earlier had indicated that a mixture of birch-alder-hazel pollen seemed to be more effective in reducing symptoms than birch pollen extract alone [9].

The efficacy and safety of SCIT was reported in a 1-year, double-blind, placebo-controlled trial monitoring clinical symptoms, medication scores and effects on allergen-specific provocation tests [10]. Another SCIT study investigated airway symptoms and medication over 2 years in a placebo-controlled treatment with the same natural birch pollen extract adsorbed onto aluminum hydroxide and also found clinical efficacy and an even better reduction of median symptom scores after the second year [11]. Very recently, the clinical efficacy, reduction in skin test responses and allergen-specific antibody responses have been analyzed in a double-blind, placebo-

controlled multicenter SCIT study comparing birch pollen extract, purified natural and recombinant major birch pollen allergen, Bet v 1 [12]. In this study, it was found that treatment with rBet v 1 was as effective and safe as treatment with birch pollen extract and induced allergen-specific IgG responses which were associated with a reduction in symptoms, medication scores and skin sensitivity [12]. An association between the reduction in nasal reactivity and the induction of Bet v 1-specific IgG antibodies was also observed in patients who had received SCIT with genetically modified hypoallergenic Bet v 1 derivatives [13]. Patients treated with the hypoallergenic Bet v 1 derivatives developed IgG responses which cross-reacted with Bet v 1-related *Fagales* and plant food allergens and were directed at new and partially sequential epitopes, not recognized before therapy [14].

In this study, we analyzed the humoral immune responses (IgE, IgG1–4 and IgA) of birch-pollen-allergic patients who had received SCIT as described in the studies by Bodtger et al. [10] and Arvidsson et al. [11] with different cumulative doses for different periods of time. Recombinant major allergens from birch (Bet v 1) and the cross-reactive allergens from alder (Aln g 1), hazel (Cor a 1), and apple (Mal d 1) were used in order to measure immune responses to defined allergen molecules. Recombinant fragments of Bet v 1 and synthetic Bet v 1-derived peptides were prepared to study the epitope specificity of the therapy-induced IgG responses. Furthermore, we developed a serological competition assay to study whether therapy-induced Bet v 1-specific IgG can block IgE binding to Bet v 1.

Material and Methods

Recombinant Allergens, Synthetic Bet v 1 Peptides, Recombinant Bet v 1 Fragments

Recombinant pollen allergens (rBet v 1, rAln g 1, rCor a 1, rMal d 1) were purchased from Biomay (Vienna, Austria). Synthetic peptides spanning the Bet v 1 sequence were synthesized on the Applied Biosystems peptide synthesizer, Model 433A (Foster City, Calif., USA) (fig. 1) [15]. Recombinant Bet v 1 fragments comprising Bet v 1 amino acids 1–74 and Bet v 1 amino acids 75–160 were expressed and purified as described (fig. 1) [16].

Patients and Blood Samples

The profile of antibody responses was studied in 12 patients allergic to birch pollen with symptomatic tree/birch pollen allergy (presence of at least one of the following symptoms: rhinitis, conjunctivitis or asthma). The demographic and clinical characteristics of the patients are summarized in table 1. The patients had received routine perennial SCIT with allergen extracts adsorbed onto aluminum hydroxide gel (Alutard SQ, ALK-Abelló,

Table 1. Demographic and clinical characteristics of patients allergic to birch pollen

No.	Sex	Age	Symptoms	Oral allergy syndrome before SCIT	Birch-pollen-specific IgE (RAST class)	SCIT with:	Pre-treatment serum	Post-treatment serum	Duration of SCIT	Classification of treatment	Cumulative dose SQ units	Birch-specific IgE kUA/l	Bet v 1-specific IgE kUA/l
1	f	27	r, c, ls	no	4	birch	21.09.2004	04.01.2005	4 months	short-term	325,540	19.1	20.2
2	m	33	r, c, ls, oas	apple, bulb, nectarine, hazelnut	4	birch	12.10.2004	04.01.2005	3 months	short-term	85,540	21.9	30.7
3	m	44	r, c, ls, oas	apple	4	birch	n.a.	04.01.2005	2 months	short-term	45,520	n.d.	31.4
4	m	38	r, c, ls, oas	walnut	n.d.	birch	n.a.	04.01.2005	11 months	short-term	800,430	n.d.	n.d.
5	m	14	r, c, oas	apple, paprika, carrot	6	birch	n.a.	04.01.2005	4 months	short-term	13,540	n.d.	>100
6	m	64	r, c, ls	apple, hazelnut	4	birch	17.09.2004	04.01.2005	4 months	short-term	145,560	20.3	n.d.
7	m	48	r, c, ls, oas	apple, nuts, gelatin	3	birch	n.a.	01.02.2005	4 years	long-term	3,915,540	n.d.	n.d.
8	f	26	r, c, ls, oas	spices	3	birch/alder/hazel	23.06.2004	01.02.2005	8 months	short-term	525,540	8.67	n.d.
9	m	40	r, c, ls	no	6	birch/alder/hazel	26.09.2002	01.02.2005	3 years	long-term	1,855,520	>100	n.d.
10	m	44	r, c	no	4	birch/alder/hazel	n.a.	01.02.2005	3 years	long-term	2,965,540	35.2	n.d.
11	m	29	r, ls, oas	apple, cherry, kiwi, banana	4	birch/alder/hazel	19.12.2002	01.02.2005	2 years	long-term	1,745,520	18.8	n.d.
12	f	63	r, c	n.d.	3	birch/alder/hazel	17.11.2000	01.02.2005	4 years	long-term	3,865,540	4.94	n.d.

f = Female; m = male; r = rhinitis; c = conjunctivitis; ls = lung symptoms; oas = oral allergy syndrome; n.d. = not done; n.a = not available.

Hørsholm, Denmark) for different periods of time in two allergy clinics (Innsbruck and Graz) in Austria. SCIT for less than 1 year was designated short-term treatment, whereas SCIT for more than 1 year was defined as long-term treatment (table 1). Serum samples were obtained as indicated in table 1 and stored at -20°C until analysis. The cumulative doses in SQ units that had been injected into each of the patients until the blood donation for the 'posttreatment serum sample' was taken are displayed in table 1. Sera were analyzed by an investigator who was blinded to the patients' clinical background.

ELISA Measurements of IgG1–4 and IgA Responses

Pre- and posttreatment sera of the SCIT-treated birch pollen-allergic patients were analyzed by ELISA for IgG1–4 and IgA responses to recombinant allergens [17]. For this purpose, ELISA plates (Nunc, Maxisorp, Roskilde, Denmark) were coated with rBet v 1, rAln g 1, rCor a 1 or rMal d 1 ($5\text{ }\mu\text{g/ml}$ in 0.1 M bicarbonate buffer, pH 9.6). Sera were diluted as follows: IgG1–4: 1:50, IgA: 1:100. Plate-bound allergens were incubated with the diluted patient sera and for control purposes with serum dilutions from a nonallergic individual. Bound antibodies were detected as described [17]. Bound IgA antibodies were detected with an alkaline-phosphatase-conjugated anti-human IgA antiserum (BD Biosciences, Franklin Lakes, N.J., USA). The color reaction was started for IgG1, IgG2, IgG3 and IgG4 antibodies by addition of 1.7 mM 2,2'-azinobis-[3-ethyl-benzthiazoline-6-sulfuric acid] (Sigma) in 60 mM citric acid, 77 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 3 mM H_2O_2 . For IgA detection, the plates were incubated with alkaline phosphatase substrate (Sigma) in 13% diethanolamine, 4 mM NaN_3 pH 9.8. Extinctions at 405 nm were determined with an ELISA reader (Dynatech, Denkendorf, Germany, or Spectramax PLUS, Molecular Devices, Sunnyvale, Calif., USA). All determinations were performed in duplicate and the results are displayed as mean values with a mean coefficient of variation of 5.3% . In order to avoid plate-to-plate variabilities, pre- and posttreatment sera were analyzed on the same plates. The percent increase or

reduction in median IgG subclass binding was determined according to the formula: $\% \text{ increase/decrease} = 100 \times \text{median OD}_{\text{Post}}/\text{median OD}_{\text{Pre}} - 100$, where OD_{Pre} represents extinctions after incubation of the coated allergen with pretreatment sera and OD_{Post} represents extinctions after incubation with posttreatment sera. The cut-offs of the separate ELISA experiments were determined according to the buffer control which reproducibly yielded OD values of approximately 0.02 on each plate.

Inhibition of Allergic Patient IgE Binding to rBet v 1 by SCIT-Induced IgG as Determined by ELISA Competition

ELISA plates (Nunc) were coated with rBet v 1 ($1\text{ }\mu\text{g/ml}$) as described before. Pre- and posttreatment sera of the SCIT-treated patients were heated for 4 h to 56°C [18] to inactivate the IgE in the sera used for inhibition. Sera were diluted 1:1 and exposed to plate-bound allergens. After washing, the plates were incubated with 1:5 diluted sera from 2 patients (A and B) allergic to birch pollen, who had not received immunotherapy. Heat inactivation was controlled by addition of serum from a nonallergic individual that did not contain IgE antibodies. Bound IgE antibodies were detected with alkaline-phosphatase-coupled mouse monoclonal anti-human IgE antibodies (PharMingen) diluted 1:1,000. Detection was performed as described above and extinctions were determined with an ELISA reader. The percentage reduction of IgE binding of allergic patients after preincubation with posttreatment sera was determined according to the formula: $\% \text{ inhibition of IgE binding} = 100 - \text{OD}_{\text{Post}}/\text{OD}_{\text{Pre}} \times 100$, where OD_{Post} represents extinctions after preincubation with post-treatment sera and OD_{Pre} represents extinctions after preincubations with pre-treatment sera, respectively.

Epitope Mapping of Allergen-Specific IgG1–4

ELISA plates (Nunc) were coated with 6 synthetic Bet v 1 peptides or 2 hypoallergenic recombinant Bet v 1 fragments ($5\text{ }\mu\text{g/ml}$) (fig. 1). Sera of the 12 SCIT-treated patients were diluted 1:50 for IgG1–4. Plate-bound antigens were incubated with the diluted

sera and the bound antibodies were detected with mouse monoclonal anti-human IgG1, IgG2 and IgG4 antibodies (PharMingen) or a mouse monoclonal anti-human IgG3 antibody (Sigma). Bound secondary antibodies were detected with a horseradish-peroxidase-labeled sheep anti-mouse antiserum (Amersham, Pharmacia). All determinations were performed in duplicate and the results are displayed as mean values with a mean coefficient of variation of 6.8%.

Statistical Methods

OD values were log-transformed and analyzed separately for the isotypes and allergens with two-factor analyses of variance (ANOVA) with a repeated-measurements factor (the within subjects factor before/after therapy) and a between subjects factor (short-term/long-term treatment). The effect of main interest was the interaction between the within subjects factor and the between subjects factor (i.e., whether the hypothesis that the difference between the pre- and posttherapy concentrations are equal for short- and long-term treatment can be rejected).

IgG concentrations for the different peptides and fragments were compared with respect to treatment duration (short vs. long) by ANOVA and linear contrasts.

Homogeneity of variances was assessed by Levene's tests and normality of residuals by Lilliefors' tests.

For all analyses, uncorrected p values below 0.05 were considered significant.

Results

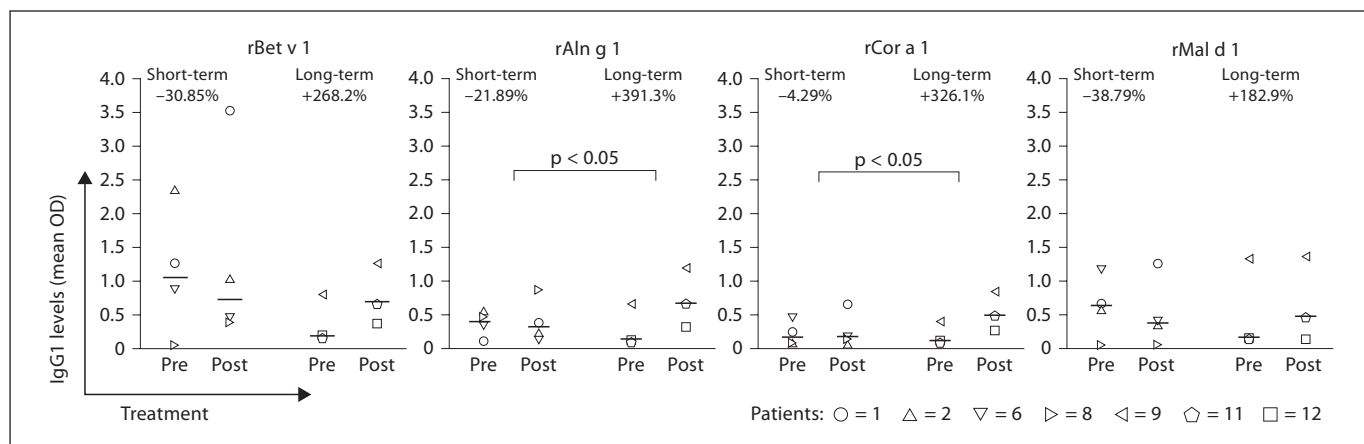
Long-Term SCIT Induced More IgG1, IgG4, IgG2 than Short-Term SCIT but No Detectable IgG3 or IgA Antibodies to Bet v 1 and Bet v 1-Related Pollen and Food Allergens

We have analyzed the IgG subclass and IgA responses to purified rBet v 1, rAln g 1, rCor a 1 and rMal d 1 in 12 patients allergic to birch pollen who had received immunotherapy with birch pollen or a birch-alder-hazel pollen extract mix (table 1). Seven of the 12 patients (patients 1–6, 8) were continuously treated for a period of less than 1 year (short-term treatment) and received cumulative doses of less than 1,000,000 SQ units, which, according to the manufacturer, corresponds to less than 120 µg of major allergen [10] (table 1). Five patients (patients 7, 9–12) were treated for more than 1 year (long-term treatment) and received a cumulative dose of more than 1,000,000 SQ units (table 1). Figures 2–5 show the IgG1, IgG2, IgG3 and IgG4 responses to ELISA plate-bound allergens from those patients whose pre- and posttreatment sera were available. Only 2 of the patients who underwent short-term treatment (i.e., patients 1, 8) developed Bet v 1-, Aln g 1- and Cor a 1-specific IgG1 responses, whereas no allergen-specific IgG1 increases were noted for the other 2 patients (patients 2, 6) (fig. 2). By contrast, we

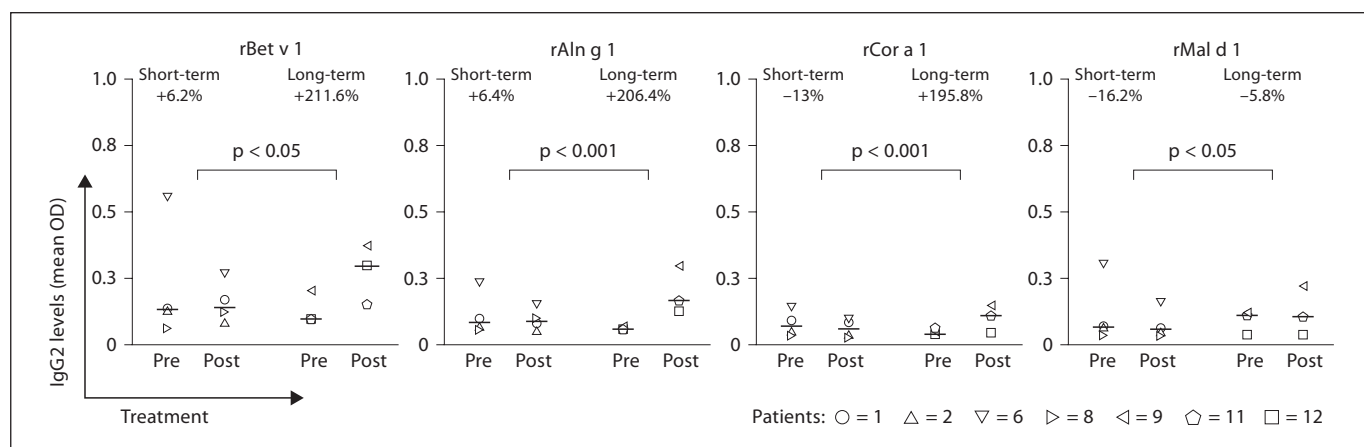
found increases in Bet v 1-, Aln g 1- and Cor a 1-specific IgG1 responses in each of the 3 patients (patients 9, 11, 12) who had been treated for more than 1 year. The IgG1 increases against Aln g 1 and Cor a 1 were significantly higher in the group on long-term treatment than in the group on short-term treatment ($p < 0.05$) (fig. 2). In general, only patients on long-term treatment developed increased median antibody responses (182.9–391.3%), whereas patients on short-term treatment even showed decreased median binding (4.29–38.79%). One of the patients on short-term treatment and one of those on long-term treatment developed IgG1 responses to the major apple allergen, Mal d 1 (patients 1, 11) (fig. 2). No relevant increases in allergen-specific IgG2 responses were found in any of the patients on short-term treatment, whereas patients on long-term treatment developed significant increases in allergen-specific IgG2 responses (increase in median Bet v 1-specific IgG2: 211.6%). These responses were primarily directed against the pollen-derived allergens (Bet v 1, Aln g 1, Cor a 1) (fig. 3). Only 1 patient (patient 9) mounted some increase in Mal d 1-specific IgG2 antibody levels after treatment (fig. 3). Neither in the patients on long-term treatment nor in those on short-term treatment were rises in allergen-specific IgG3 antibody levels found (fig. 4).

Patients on short-term treatment showed little induction of allergen-specific IgG4 (fig. 5). All 3 patients on long-term treatment (patients 9, 11, 12) increased their IgG4 levels to Bet v 1, Aln g 1 and Cor a 1 (Bet v 1: 102.1%, Aln g 1: 727.5%, Cor a 1: 348.2%). The Aln g 1- and Cor a 1-specific IgG4 increases were significantly higher in the group on long-term treatment than in the group on short-term treatment ($p < 0.001$). Only 1 patient (patient 9) increased his Mal d 1-specific IgG4 antibodies (fig. 5).

Thus IgG responses developed more frequently to the pollen-derived allergens which show greater sequence identity to each other: according to Swissprot entries, there is 81% sequence identity between Bet v 1 [3] and Aln g 1 [19]; the identity between Bet v 1 and Cor a 1 [20] is 72%. Specific IgG responses to the apple allergen Mal d 1 were rare since Mal d 1 has lower sequence identity to pollen allergens (percent identity between Bet v 1 and Mal d 1: 56%) [21]. No relevant therapy-induced induction of allergen-specific IgA (fig. 6) was found in any of the patients. Similar results were obtained by Western blot experiments using nitrocellulose-blotted natural birch pollen extract (data not shown).



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Fig. 2–5. Scatter plot representation of IgG subclass reactivities to Bet v 1 and Bet v 1-related allergens before (Pre) and after (Post) short-term and long-term SCIT. Serum IgG1 (**2**), IgG2 (**3**), IgG3 (**4**) and IgG4 (**5**) levels specific to rBet v 1, rAln g 1, rCor a 1 and rMal d 1 are shown for short-term SCIT (patients 1, 2, 6, 8) or long-

term SCIT (patients 9, 11, 12) before and after SCIT. Median OD values are displayed as black lines and the percent increases or decreases of the median OD values before and after SCIT are displayed. Statistically significant differences between short-term and long-term SCIT before and after treatment are indicated.

Posttreatment Sera from Patients on Long-Term Treatment Inhibit IgE Binding to Bet v 1 More Strongly than Sera from Patients on Short-Term Treatment

Next we investigated whether preincubation of Bet v 1 with posttreatment serum samples can inhibit IgE reactivity to the allergen. For this purpose, we subjected available pre- and posttreatment serum samples to heat-inactivation in order to remove endogenous IgE reactivity. Bet v 1 was then preincubated with those serum samples, washed, and then exposed to sera from 2 Bet v 1-allergic patients. The experiment was thus designed to study whether immunotherapy induces Bet v 1-specific antibodies belonging to Ig classes other than IgE which can inhibit the binding of birch pollen-allergic patients' IgE

to Bet v 1. Low or no inhibition (0–30%) of IgE reactivity to Bet v 1 was observed when serum samples from patients on short-term treatment were used (table 2). A much stronger inhibition of IgE binding to Bet v 1 (52–72%) was obtained with posttreatment serum samples from patients on long-term treatment (table 2).

Patients on Long-Term SCIT Mount Distinct IgG Subclass Reactivities to Recombinant and Synthetic Bet v 1 Fragments

Untreated birch-pollen-allergic patients do not mount IgE and hardly mount IgG responses to recombinant Bet v 1 fragments comprising amino acids 1–74 and amino acids 75–160 of Bet v 1 or to the synthetic Bet v 1-derived peptides described in Material and Methods (fig. 1) [15,

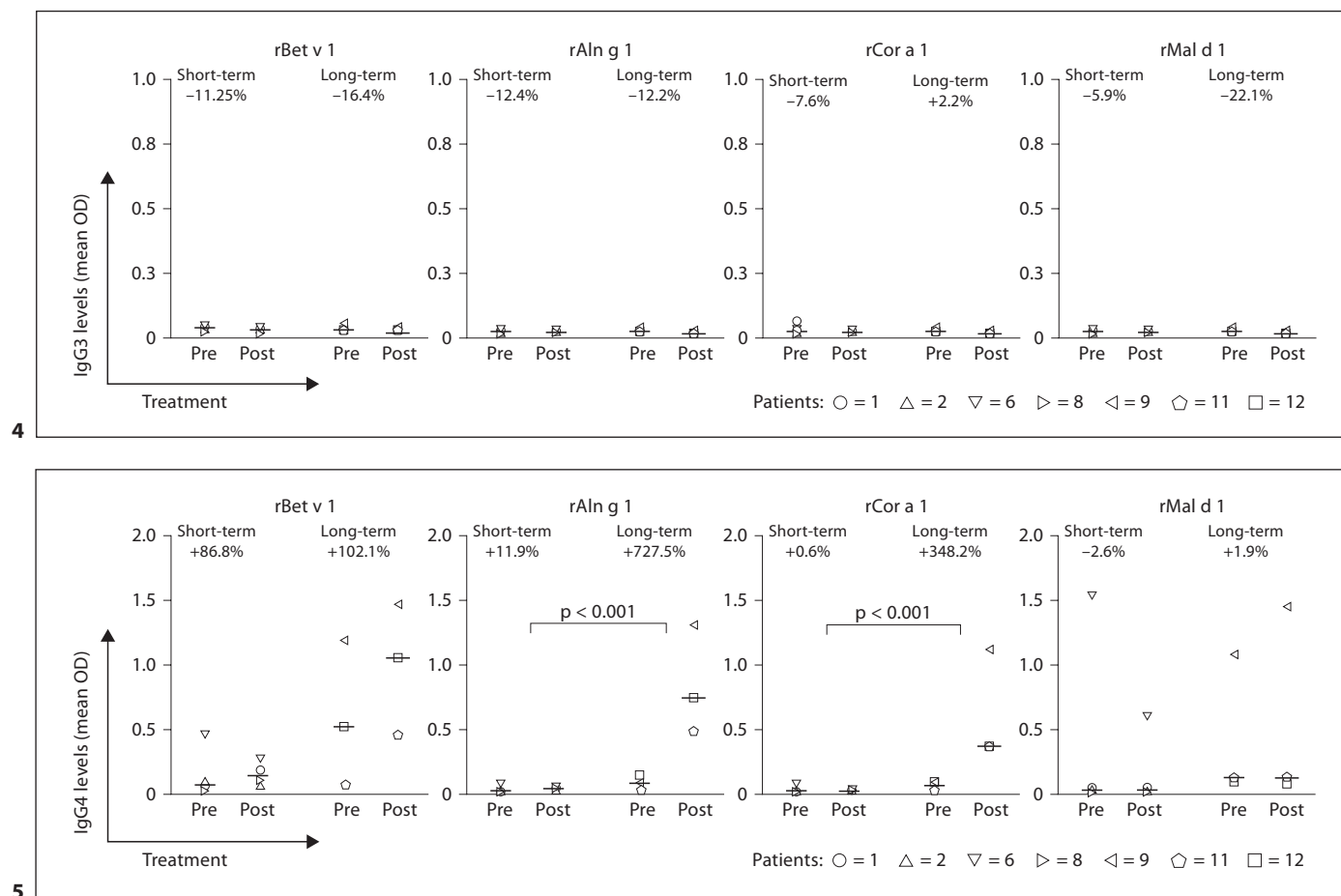


Fig. 2–5. Scatter plot representation of IgG subclass reactivities to Bet v 1 and Bet v 1-related allergens before (Pre) and after (Post) short-term and long-term SCIT. Serum IgG1 (2), IgG2 (3), IgG3 (4) and IgG4 (5) levels specific to rBet v 1, rAln g 1, rCor a 1 and rMal d 1 are shown for short-term SCIT (patients 1, 2, 6, 8) or long-

term SCIT (patients 9, 11, 12) before and after SCIT. Median OD values are displayed as black lines and the percent increases or decreases of the median OD values before and after SCIT are displayed. Statistically significant differences between short-term and long-term SCIT before and after treatment are indicated.

16]. Figure 7 displays box plot representations of the IgG1–4 subclass responses to Bet v 1 peptides and fragments in all posttreatment samples from the patients on short-term treatment (fig. 7a: 1–6, 8) and those on long-term treatment (fig. 7b: 7, 9–12). We found statistically significant rBet v 1 fragment-specific IgG1 and IgG4 responses as well as peptide-specific IgG1 and IgG4 responses mainly in the patients on long-term treatment (fig. 7b). Interestingly, we found specific IgG2 antibodies to the N-terminal peptide 1, but no IgG1 or IgG4 responses in long-term-treated patients. On the other hand, we noted that the C-terminal peptide 5 reacted only with IgG1 and IgG4 but not with IgG2 antibodies (fig. 7b). Greater IgG1 responses to recombinant Bet v 1 fragments were found in the group on long-term treatment than in

the group on short-term treatment (fig. 7a, b). IgG4 antibodies to both rBet v 1 fragments and IgG2 against the C-terminal fragment F2 were only detectable in the group on long-term treatment (fig. 7a, b).

Discussion

In this study we analyzed IgE, IgG subclass and IgA antibody responses to purified recombinant allergens as well as to recombinant fragments and synthetic peptides derived from the major birch pollen allergen, Bet v 1, in a group of patients allergic to birch pollen who had received SCIT with birch pollen or a mixture of birch-alder-hazel pollen extracts.

Fig. 6. Box plot representation of IgA reactivity to rBet v 1 before and after short-term SCIT (patients 1, 2, 6, 8; **a**) and long-term SCIT (patients 9, 11, 12; **b**). Boxes include 50% of the values and nonoutliers are located between the bars. Lines within the boxes denote median values. The cut-off is indicated by a dashed line.

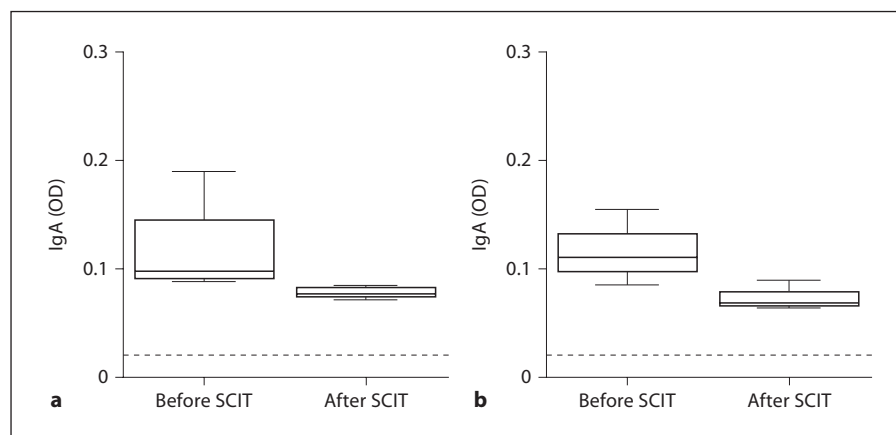


Table 2. Inhibition of IgE reactivity to Bet v 1 with posttreatment sera

	Patient A			Patient B		
	IgE binding (pretreatment sera) (OD)	IgE binding (posttreatment sera) (OD)	inhibition of IgE binding %	IgE binding (pretreatment sera) (OD)	IgE binding (posttreatment sera) (OD)	inhibition of IgE binding %
Mean short-term treatment	0.59	0.5	14.7	0.39	0.39	0
Serum 1	0.68	0.59	13	n.d.	n.d.	n.d.
Serum 2	0.59	0.58	1	0.41	0.41	0
Serum 8	0.49	0.34	30	0.37	0.37	0
Mean long-term treatment	0.38	0.13	65	0.32	0.1	71
Serum 9	0.4	0.12	71	0.33	0.1	71
Serum 11	0.37	0.18	52	n.d.	n.d.	n.d.
Serum 12	0.38	0.1	72	0.31	0.09	71

Inhibition of IgE reactivity to Bet v 1 with posttreatment sera. ELISA plate-bound Bet v 1 had been preincubated with pretreatment and posttreatment sera from patients 1, 2, 8, 9, 11, 12 and was subsequently exposed to sera from 2 other patients allergic to birch pollen, A and B. The OD values corresponding to IgE reactivity of patients A and B to Bet v 1 after preincubation with pre- and posttreatment sera and the corresponding percentages of inhibition are displayed. n.d. = Not done.

As in previous SCIT studies where we have analyzed the antibody responses of patients who had received SCIT with grass pollen extracts [22], we found an induction of allergen-specific IgG responses, particularly in the IgG1 and IgG4 subclass, a low but detectable induction of Bet v 1-specific IgG2, but no detectable Bet v 1-specific IgG3 or IgA responses. Analysis of the antibody responses to Bet v 1-related allergens from alder (*Aln g 1*), hazel (*Cor a 1*) and apple (*Mal d 1*) showed that immunotherapy with allergen extracts induced IgG responses of varying intensities to the cross-reactive allergens. There are several possibilities for this varying induction of IgG responses to Bet v 1 and the Bet v 1-related allergens. They include

different genetically determined abilities of the patients to mount immune responses [23], different degrees of cross-reactivity among the Bet v 1-related allergens [24, 25] and possible heterogeneities of the raw allergen extracts used for treatment [26–28].

However, the fact that vaccines which have been formulated with defined amounts of purified recombinant Bet v 1 or genetically modified Bet v 1 derivatives have induced robust IgG responses to Bet v 1 and Bet v 1-related allergens may rather point to weaknesses regarding the manufacturing process of the extracts [12, 14]. It has been shown that immunotherapy with birch pollen extract and recombinant birch pollen allergen, Bet v 1 and

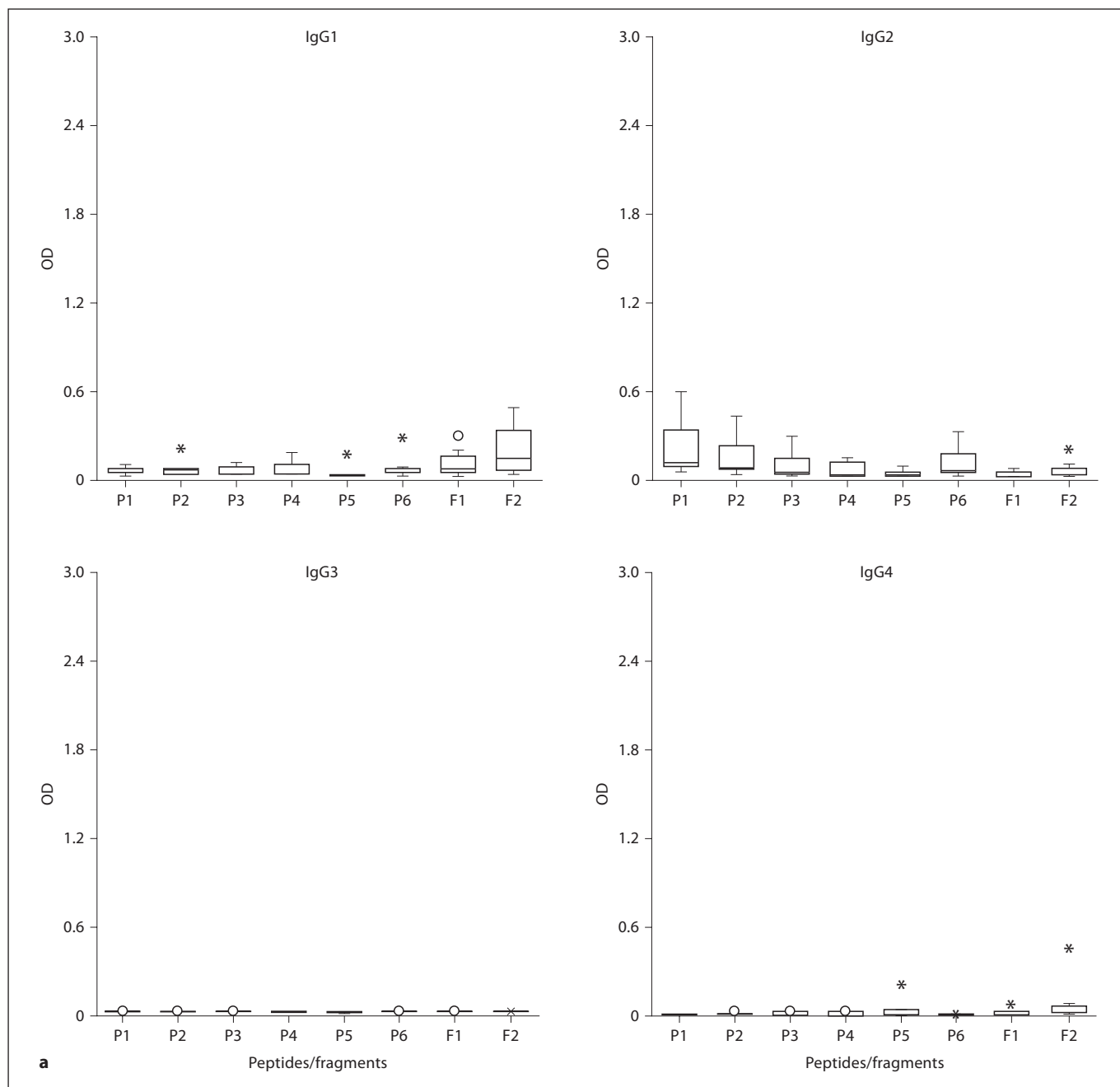
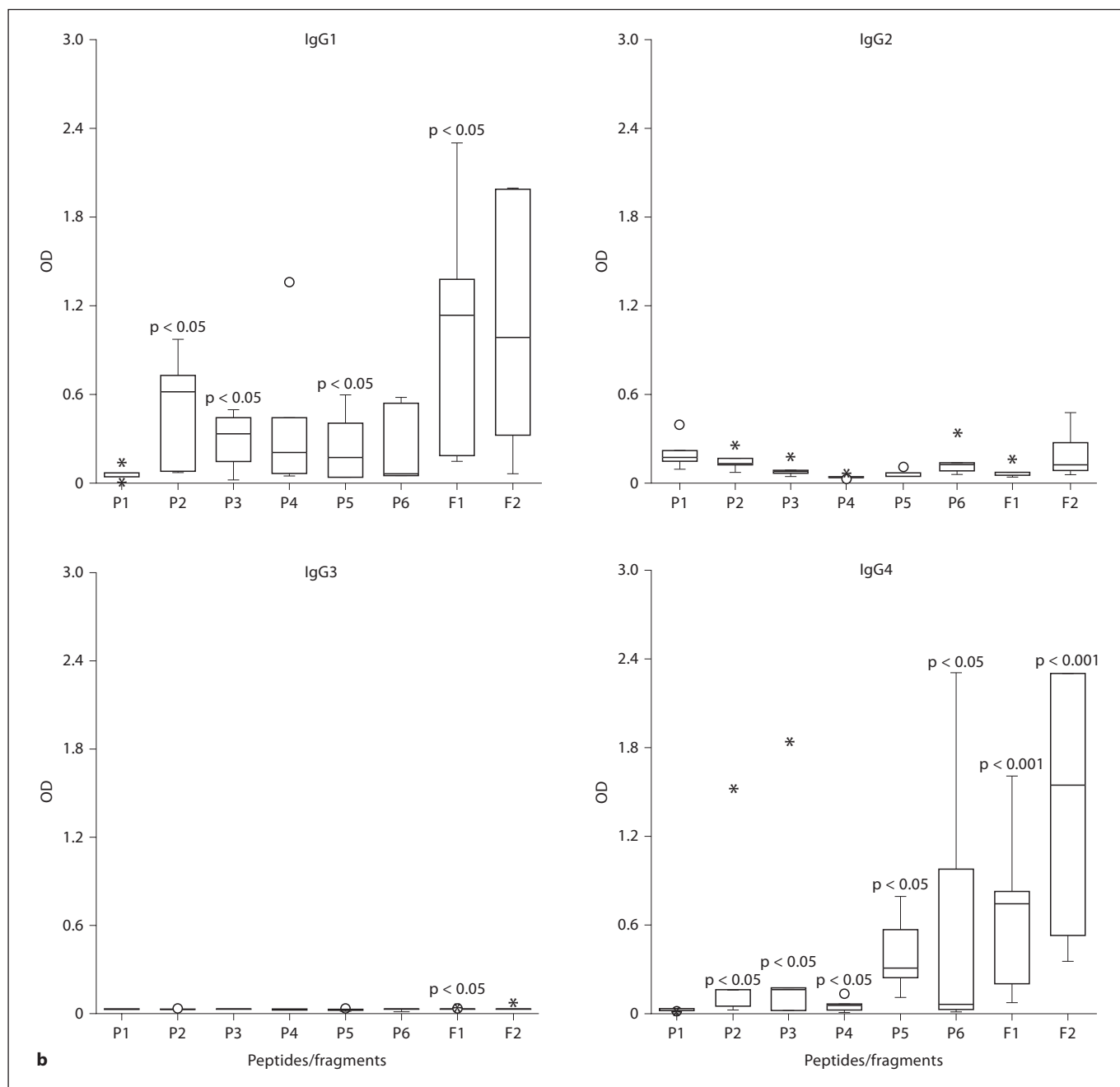


Fig. 7. Epitope mapping of IgG subclass responses in posttreatment sera following short-term SCIT (patients 1–6, 8; **a**) and long-term SCIT (patients 7, 9–12; **b**). IgG1, IgG2, IgG3 and IgG4 levels specific to Bet v 1-derived peptides (P1–P6) and fragments (F1, F2) are displayed as box plots where 50% of the values are within

the boxes and nonoutliers between the bars. Lines within the boxes denote median values. Open circles and asterisks indicate outliers and extremes. **b** p values are indicated when there were statistically significant differences between long-term and short-term SCIT.

rBet v 1 derivatives induces protective IgG responses against the cross-reactive *Fagales* pollen and plant food allergens and may improve allergic symptoms to the birch-pollen-related allergen sources [6–8, 14, 29–31]. In

particular, injection immunotherapy trials with adjuvant-bound purified recombinant birch pollen allergen Bet v 1 and with genetically modified derivatives of Bet v 1 have demonstrated that treatment with purified Bet



v 1 induces highly specific, robust and protective IgG responses not only to natural Bet v 1 [32], but also to Bet v 1-cross-reactive plant allergens [14, 31].

In fact, recent studies have shown that the contents of the major birch pollen allergen Bet v 1 may indeed vary up to tenfold in commercially available birch pollen extracts [33]. It is therefore possible that the heterogeneity of the immune responses in our patients is due to the het-

erogeneity of the allergen extracts used to treat the patients in our study. In addition to this explanation, two other factors must be considered: First, we found that patients who had received treatment for longer than 1 year mounted higher allergen-specific IgG responses than patients who were treated for less than 1 year. Second, patients on long-term treatment had also received higher cumulative doses of major allergens than those on short-

term treatment, which may also affect the levels of IgG production. In this context, it should be noted that a double-blind, placebo-controlled SCIT study performed with allergen extracts similar to those used in our patients reported a stronger reduction in symptoms after the second year of treatment compared with the first year [11].

As in previous immunotherapy studies performed with grass pollen allergen extracts, we could not detect any relevant allergen-specific IgA responses after treatment [22].

Interestingly, we found that long-term treatment in contrast to short-term treatment induced IgG1, IgG4 and IgG2 responses against synthetic Bet v 1-derived peptides as was also the case for SCIT with genetically modified rBet v 1 derivatives [32]. These therapy-induced IgG responses were directed against epitopes which were not recognized in untreated patients and distinct from IgE epitopes because we did not find any IgE responses against these Bet v 1 peptides in untreated patients (data not shown) [14–16, 32]. Nevertheless, using a simple ELISA competition assay, we found that therapy-induced IgG antibodies blocked IgE reactivity to the major birch pollen allergen Bet v 1. The degree of inhibition of IgE reactivity was associated with the levels of Bet v 1-specific IgG antibodies. This finding is in agreement with mechanistic studies performed in experimental animal models [34, 35] and with results obtained in immuno-

therapy studies performed with purified allergens [14]. Our findings together with observations made in studies performed with purified recombinant allergens thus strongly support the concept that SCIT induces an allergen-specific counter-IgG response which competes with IgE for allergen binding [12, 14, 32, 36]. The counter-IgG responses obviously do not need to be directed exactly against the epitopes that are recognized by IgE because any reactivity in the vicinity of IgE epitopes or the simple occupation of certain amino acid residues required for the interaction with IgE will lead to steric hindrance of IgE binding. As a consequence of the inhibition of the IgE recognition of allergens, a reduction in allergen-induced immediate-type and late-phase reactions can be expected and may be a major mechanism of SCIT [37–39].

Our results thus show that recombinant allergens and allergen-derived epitopes are useful tools for monitoring SCIT and stress the importance of blocking antibodies during SCIT.

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