

Birch pollen immunotherapy results in long-term loss of Bet v 1-specific T_H2 responses, transient T_R1 activation, and synthesis of IgE-blocking antibodies

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Background: Early events of specific immunotherapy (SIT) are induction of allergen-specific IL-10-producing T_R1 cells and production of IgG antibodies, but there is little knowledge about the long-term immune mechanisms responsible for sustained allergen tolerance.

Objective: Bet v 1-specific immune responses of 16 patients with birch pollen allergy were characterized up to 54 months at defined time points before, during, and after a 3-year period of SIT.

Methods: We sought to analyze allergen-specific T- and B-cell responses. Bet v 1-specific IL-5-, IFN- γ -, and IL-10-secreting T cells were quantified in peripheral blood, and birch pollen-specific IgE and IgG antibody levels were determined in serum. Furthermore, the inhibitory capacity of SIT-induced IgG was evaluated by blocking allergen binding to IgE and inhibition of facilitated allergen presentation.

Results: Seasonal increases in Bet v 1-specific T_H2 cell numbers ceased to appear after the first year of SIT without deviation to a T_H1-dominated immune response. Furthermore, the frequency of IL-10-producing T_R1 cells, which had increased during the first year of SIT, returned to pretreatment levels in the second year. In contrast, allergen-specific IgG antibody concentrations continuously increased during SIT but started to decrease after cessation of treatment. Functional analysis confirmed the ability of the IgG antibodies to inhibit IgE-allergen interactions, which peaked at the end of SIT but then slowly started to decrease. **Conclusion:** Long-term allergen tolerance achieved by SIT is associated with the development of peripheral T-cell tolerance characterized by decreased reactivity of Bet v 1-specific T_H2 cells and enriched allergen-specific IgG competing with IgE antibodies for allergen binding. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Respiratory allergy, specific immunotherapy, allergen tolerance, Bet v 1, IgE, IgG, blocking antibodies, regulatory T cells

The long-lasting clinical efficacy of specific immunotherapy (SIT) has been well documented,¹⁻⁵ but the underlying immune mechanisms are still a focus of intensive research. Thus far, most immunologic studies have concentrated on the development of allergen tolerance during the first 1 to 2 years of SIT. Initially, a shift from an allergen-specific T_H2 to a T_H1 immune response, resulting in the correction of an imbalance between T_H2 and T_H1 cells in allergic subjects, was regarded as an important mechanism of successful SIT.⁶⁻⁸ Later, it appeared that another T-cell subset called regulatory T (Treg) cells plays a major role in the development of allergen tolerance.⁹ We and others could show that IL-10-producing type 1 Treg (T_R1) cells, which exhibit distinct features discriminating them from CD4⁺CD25⁺CD127^{low/-} forkhead box protein 3 (Foxp3)⁺ Treg cells,¹⁰⁻¹⁴ are induced during the first few months of SIT.^{13,15,16} In addition, an increased production of allergen-specific IgG, especially IgG₄, antibodies was noticed early on during SIT.^{15,17-19} Because these antibodies were able to block allergen-IgE interactions *in vitro*, a functional role in inhibiting IgE-mediated allergic reactions was suggested.²⁰

Although these observations helped to elucidate mechanisms of how SIT promotes the induction of allergen tolerance, data about immunologic alterations associated with long-term efficacy of SIT, in particular covering the time after its discontinuation, are very sparse. Of note, to our knowledge, there are no reports of longitudinal studies about the potential role of allergen-specific T-cell subsets in sustaining SIT-induced allergen tolerance beyond the treatment period. Only a few studies thoroughly analyzed humoral immune responses by following allergen-specific antibody concentrations in patients who completed either subcutaneous or sublingual SIT with aeroallergens.²¹⁻²⁴ Although all showed increased IgG₄ serum concentrations, correlation with the clinical outcome was inconsistent, raising the question of whether other immunologic markers, such as the functional ability of these antibodies to block allergen-IgE interaction, are better suited to assess the efficacy of SIT^{20,22} and pointing to the need for clinical studies more closely investigating the immune alterations underlying the long-term outcome of allergic patients treated by means of SIT.

METHODS

Patients

The investigated cohort consisted of 16 patients with birch pollen allergy. Inclusion/exclusion criteria and detailed SIT-induced alterations during the first year of therapy of all but 1 patient were previously published.¹³ Briefly, participants had a history of moderate-to-severe birch

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Abbreviations used

FAP: Facilitated allergen presentation
 Foxp3: Forkhead box protein 3
 PMP: Paramagnetic particle
 SIT: Specific immunotherapy
 SPT: Skin prick test
 Treg: Regulatory T

pollen-induced rhinoconjunctivitis and/or mild-to-moderate asthma, positive skin prick test (SPT) and nasal provocation responses to birch pollen extract (ALK Prick SQ and ALK-depot SQ, respectively; both from ALK-Abelló, Hørsholm, Denmark), and specific serum IgE reactivity against birch pollen extract and rBet v 1 (Phadia ImmunoCAP System; Phadia, Uppsala, Sweden). Patients with a history of clinically relevant allergies against other pollens, polysensitization with perennial symptoms, or chronic nonallergic asthma were excluded. Withdrawal of subjects occurred because of non-treatment-related events. The study was approved by the Ethics Committee of the Medical Faculty of Philipps University, Marburg, Germany; all patients provided written informed consent to participate in the trial.

Study design

Details of the SIT protocol have been reported previously.¹³ In brief, patients received weekly up-dosing injections subcutaneously until the maintenance dose of 100,000 standard quality units of birch pollen allergen per injection was reached, followed by monthly maintenance injections for 3 years.

Further details on evaluation of birch pollen extract SPT reactivity and assessment of SIT-treated patients' symptoms during natural birch pollen exposure are provided in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Blood samples and analysis of PBMCs

Preparation of peripheral blood samples and subsequent characterization of PBMCs by means of ELISpot and ELISA analysis are described in detail in the [Methods](#) section in this article's Online Repository.

Analysis of allergen-specific serum immunoglobulins

Levels of total IgE, specific IgE, IgG and IgG₄ antibodies against *Betula verrucosa* (birch) pollen allergen, and specific IgE antibodies against rBet v 1 were measured by using the Phadia ImmunoCAP System, according to the manufacturer's instructions. In addition, specific IgG₁ and IgG₄ antibodies against birch pollen extract and nBet v 1 and rBet v 1 were determined by means of Western blotting (see additional information in the [Methods](#) section in this article's Online Repository).

For further details on facilitated allergen presentation (FAP), IgE blocking factor assay, and depletion of IgG antibodies, see the [Methods](#) section in this article's Online Repository.

Statistical analysis

Comparison of paired samples with at least 6 patients before and at different time points during/after SIT was done by using the nonparametric Wilcoxon signed-rank test with SPSS 17.0 software (SPSS, Chicago, Ill). Differences were considered statistically significant at *P* values of less than .05, less than .01, and less than .001.

RESULTS**Sustained clinical efficacy of birch pollen SIT**

Clinical efficacy of SIT was assessed by reporting the extent of allergic symptoms during natural birch pollen exposure and

determining the SPT reactivity to birch pollen extract. Amelioration of allergic symptoms, which was already achieved during the first SIT year,¹³ persisted over the entire treatment period and the 2 years thereafter ([Table I](#)). In addition, SPT reactivity was significantly decreased during SIT but returned to pretreatment values 2 years after stopping therapy in the follow-up subgroup ([Table I](#)).

Long-term loss of Bet v 1-specific T_H2 cell responses

Compared with the first SIT year, when numbers of Bet v 1-specific IL-5-secreting T cells still increased because of natural allergen exposure (month 6), a markedly reduced allergen-specific T_H2 cell response was noticed during the birch pollen seasons in the second and third SIT years (months 18 and 30) and in the subsequent 2 years after termination of SIT (m42 and m54; [Fig 1](#)). Although Bet v 1-specific IFN- γ -producing T-cell numbers also significantly decreased during active treatment ([Fig 1](#)), their frequency increased again after completion of SIT, reflecting the condition seen in healthy control subjects during the birch pollen season.¹³

Transient induction of Bet v 1-specific T_R1 cells

As previously reported, Bet v 1-specific T cells, characterized as T_R1 cells by suppression of T effector cell proliferation in an IL-10-dependent manner and the lack of Foxp3 expression, were induced 3 months after SIT initiation, and their numbers continued to increase until the first birch pollen season (month 6; [Fig 1](#)).¹³ However, allergen-specific T_R1 cell activation returned to pretreatment levels despite ongoing birch pollen immunotherapy for another 2 years. Thus clinical improvement was sustained in SIT-treated patients despite an only temporary activation of Bet v 1-specific T_R1 cells.

Prolonged decrease of IL-5 and IL-10 secretion by PBMCs

Although increased frequencies of the above-described Bet v 1-specific T-cell subsets were not detected by using ELISpot analysis after the first SIT year, we determined the secretion of the respective cytokines (IL-5, IFN- γ , and IL-10) by analyzing the supernatants of Bet v 1-stimulated PBMCs. Compared with pretreatment levels, IL-5 and IL-10 concentrations were enhanced in the first birch pollen season (month 6; see [Fig E1](#) in this article's Online Repository at www.jacionline.org) but then continuously decreased until the end of the follow-up period. In addition, IFN- γ levels were increased during neither the SIT nor the post-SIT periods (see [Fig E1, B](#)). Thus the cytokine ELISA profile of PBMCs from SIT-treated patients confirmed the loss of a Bet v 1-specific T_H2 cell response after the first year of SIT, the absence of a T_H1 cell shift, and the transient activation of Bet v 1-specific T_R1 cells, respectively.

Alterations of birch pollen-specific immunoglobulin concentrations

Both birch pollen-specific and Bet v 1-specific IgE levels did not change significantly during SIT or in the subsequent 2 years thereafter (see [Fig 2, A](#), and [Fig E2](#) in this article's Online

TABLE I. Clinical characteristics of patients with birch pollen allergy

	Before SIT	SIT			After SIT	
	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
Clinical score						
In season	Month -6	Month 6	Month 18	Month 30	Month 42	Month 54
No.	15	15	12	12	8	6
Median (range)	5 (3-6)	3** (1-5)	2** (1-4)	3* (1-5)	3* (1-3)	3.5 (1-5)
SPT						
Out of season	Month 0	Month 12	Month 24	Month 36		Month 60
No.	16	14	11	10		6
Median (range)	2 (1-4)	1** (0.5-2)	1** (0.5-1)	1** (0.5-1)		2 (1-2)
Total IgE (kU/L)	Month 0			Month 30		Month 54
No.	16			12		7
Median (range)	103.5 (3.08-1040)			96.35 (2-830)		155 (40.8-786)

Patients undergoing SIT: n = 16; sex (female/male): 10/6; age (median [range]): 43 years (21-61 years).

* $P < .05$ and ** $P < .01$ compared with values before initiation of SIT, as determined by using the Wilcoxon matched-pairs signed-rank test.

Repository at www.jacionline.org). In contrast, a continued increase in allergen-specific IgG levels was noticed during the 3 years of SIT (Fig 2, B). However, although allergen-specific IgG concentrations remained increased during the following 2 years, they started to decrease after termination of SIT. Of note, allergen-specific IgG₄ levels showed a parallel course in SIT-treated patients (Fig 2, C). Likewise, the ratio of birch pollen-specific IgE to IgG (Fig 2, D) and IgG₄ (see Fig E3 in this article's Online Repository at www.jacionline.org) levels decreased significantly during SIT and remained less than pretreatment values, although these levels started to increase after SIT termination.

Monitoring the patients' sera more closely by means of Western blotting, increases in allergen-specific IgG levels were related to not only Bet v 1-specific IgG₄ but also IgG₁ antibody levels (Fig 3 and see Fig E4 in this article's Online Repository at www.jacionline.org). Furthermore, levels of both IgG₁ and IgG₄ decreased after cessation of SIT in a time-dependent manner.

SIT-dependent inhibition of IgE-mediated FAP

It has been suggested that allergen-specific IgG serum antibodies can block the interaction between allergen and IgE antibodies, thereby preventing IgE-mediated immune reactions.^{25,26} Because Bet v 1-specific IgG antibody concentrations started to decrease after completing SIT, the potential of SIT-treated patients' sera to inhibit allergen-IgE complex formation was analyzed by using the FAP assay before, during, and after finishing SIT. Mean FAP was significantly inhibited in the first SIT year, with some patients exhibiting steep inhibition curves and others showing a more gradual increase of FAP inhibition (Fig 4, A). Apparent FAP inhibition persisted until 6 months after cessation of SIT; however, in the vast majority of patients, serum-induced FAP inhibition already started to diminish at this time point.

SIT-induced blocking activity of allergen-IgE interaction

The time course of SIT-induced serum capacity to inhibit allergen-IgE binding was confirmed by determining the ratio between IgE-allergen interactions measured in the presence and absence of patients' sera gathered at different time points. The results shown in Fig 4, B, demonstrate that similar to FAP

inhibition after 12 months of SIT, a significant allergen-specific IgE-blocking effect was observed. Except for month 18, this effect was sustained during SIT; however, after SIT, most patients lost the blocking effect, with mean values finally even returning to pre-SIT values. Thus although SIT results in a continuous increase in allergen-specific blocking activity, this activity starts to diminish after finishing SIT.

IgE-blocking activity is mediated by allergen-specific IgG antibodies

An inverse relation between SIT-induced allergen-specific IgG concentrations and the IgE blocking factor was observed (Fig 5, A). Therefore the potential role of IgG in blocking IgE-allergen interaction was elucidated more closely by depleting allergen-specific IgG levels in plasma of a subgroup of 5 SIT-treated patients who showed enhanced concentrations of birch pollen-specific IgG (Fig 5, B). As shown in Fig 5, C, the depletion of birch pollen-specific IgG led to an abrogation of the blocking capacity in the plasma samples of 4 of 5 SIT-treated patients. Interestingly, the patient with the highest IgE/IgG₄ ratio showed no allergen-specific IgE blocking of the analyzed samples. Thus SIT resulted in a long-term and apparently T cell-independent production of Bet v 1-specific IgG antibodies able to block IgE-allergen binding.

DISCUSSION

SIT-induced allergen tolerance is initiated by using decisive immunologic mechanisms (ie, the development of IL-10-secreting T_R1 cells and the synthesis of allergen-specific IgG antibodies, both of which are observed during the first year of treatment).^{6,13,15} Monitoring our previously published cohort of patients with birch pollen allergy¹³ for another 4 years, we here show that the maintenance and posttreatment phases of SIT are associated with distinct allergen-specific cellular and humoral immune alterations. Thus our results emphasize a dynamic process in both establishing and sustaining allergen tolerance, which is orchestrated by a distinct interplay of different immune parameters. The most important findings are (1) a loss of T_H2 cell reactivity on Bet v 1 stimulation from the second treatment year on, which is maintained even after termination of SIT; (2) a transient induction of Bet v 1-specific IL-10-producing T_R1 cells; and (3) a continued

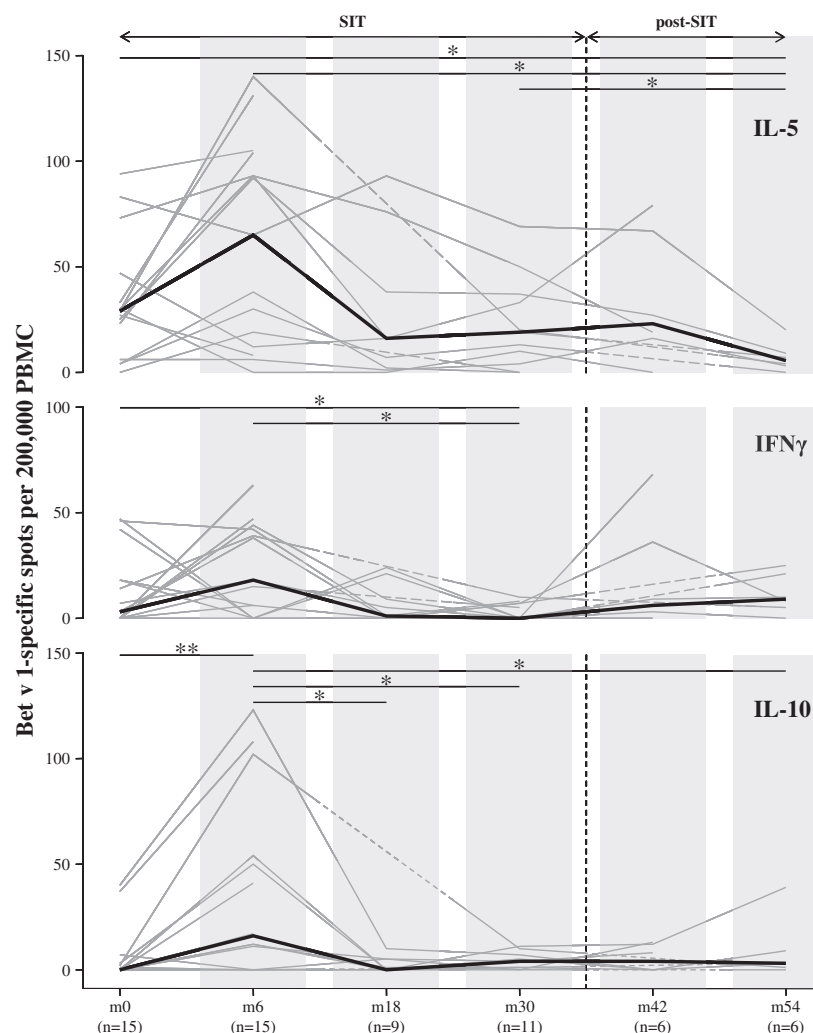


FIG 1. ELISpot analysis of Bet v 1-specific IL-5-, IFN- γ -, and IL-10-producing T cells during a 54-month (m0-m54) observation period including SIT with birch pollen extract and a follow-up phase after cessation of treatment (post-SIT). Median values are indicated by *black lines*. Birch pollen seasons are highlighted in *gray*. Discontinuities by *dashed lines* mark nonexistent data. Differences between initiation of SIT and during/after SIT were analyzed by using the nonparametric Wilcoxon signed-rank test. * $P < .05$ and ** $P < .01$.

increase of allergen-specific blocking IgG levels (ie, IgG₁ and IgG₄) during the maintenance phase of SIT, followed by subsequent progressive decreases in both specific IgG concentrations and allergen-blocking activity in patients' sera after finishing SIT.

Different T cell-mediated immune mechanisms have been proposed to be the basis of allergen tolerance. Because IgE-mediated allergies are thought to be T_H2 cell driven, it has been suggested that SIT leads to a shift from a T_H2 to a T_H1 cell-dominated immune response.^{7,8,27} Indeed, we observed a sustained loss of Bet v 1-specific T_H2 cell reactivity in SIT-treated patients after the first year of therapy, but this was not accompanied by an increase in allergen-specific T_H1 cell numbers. More recent data point toward Treg cells as key players in the establishment of allergen tolerance,^{28,29} with particular focus on IL-10-secreting T_R1 cells, which are induced early on during SIT.^{6,15,16} However, analyzing both the frequencies of allergen-specific T_R1 cells and IL-10 secretion by PBMCs for up to 5 years after starting SIT, our data suggest that Bet v 1-specific T_R1 cell activation is rather a transient event. Although we cannot exclude that allergen-

specific T_R1 cells might have shifted to effector organs to mediate allergen tolerance locally rather than systemically,^{30,31} the SIT-induced Treg cell response might be important for the initiation but not for the maintenance of allergen tolerance. This is substantiated by other studies showing that the frequency of CD4⁺CD25⁺Foxp3⁺ Treg cells, IL-10 expression, or both increased early on during SIT with aeroallergens^{6,32,33} or oral desensitization with peanuts³⁴ but later on decreased³³ and mostly returned to pretreatment levels in the course of SIT, even though clinical improvement persisted.^{6,32,34}

The disappearance of Bet v 1-specific T-cell reactivity suggests that long-term preservation of allergen tolerance might not rely on active T-cell contribution but rather be due to diminished allergen-specific T-cell responses in general, resembling the immunologic phenotype of healthy nonallergic subjects.^{13,29,35,36} Different mechanisms could be responsible for this, such as SIT-induced T-cell depletion, induction of T-cell anergy, or development of allergen ignorance.^{27,37-39} For example, a recent study suggested that preferential depletion of terminally

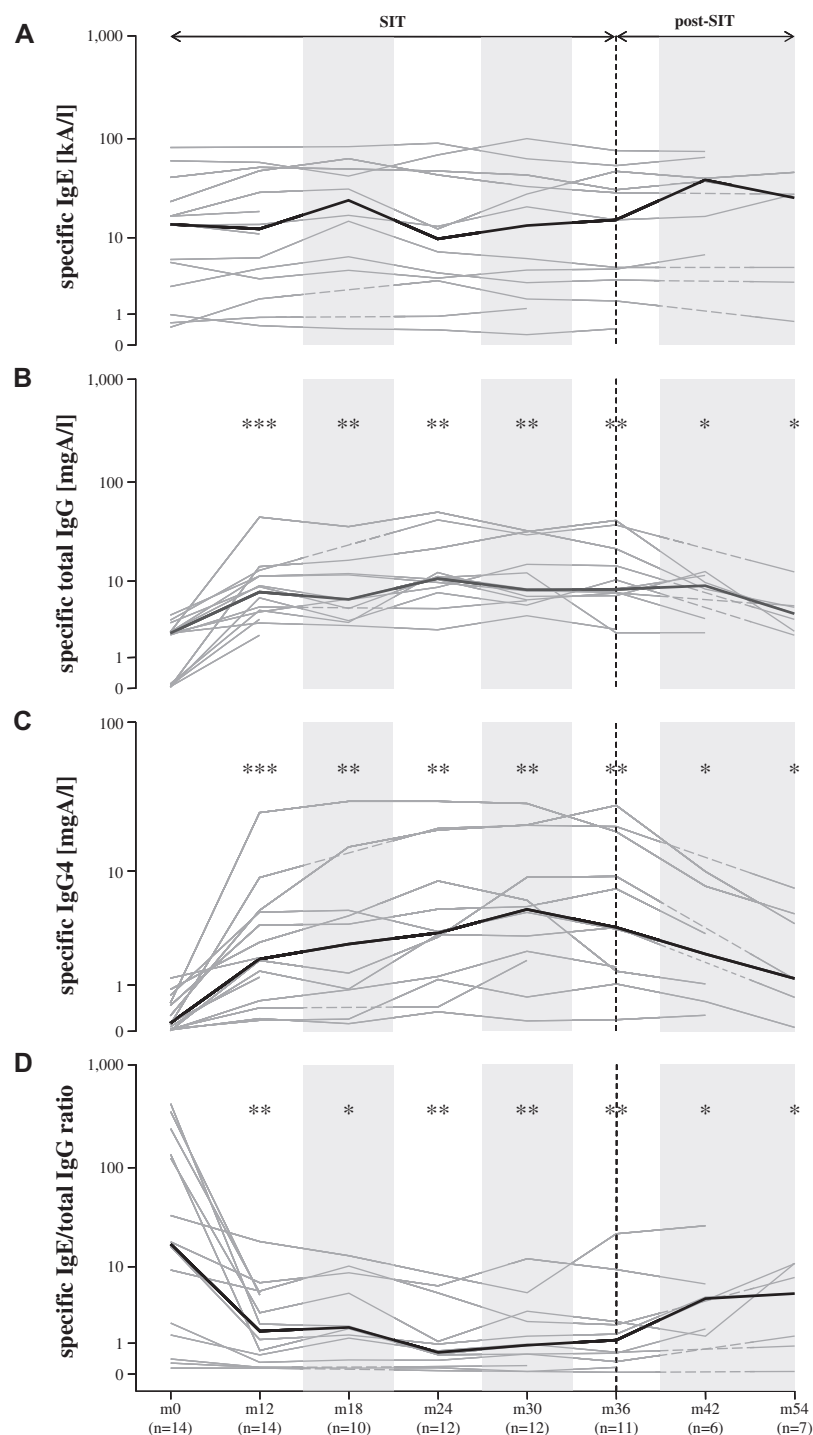


FIG 2. A–C, Birch pollen-specific IgE (Fig 2, A), total IgG (Fig 2, B), and IgG₄ (Fig 2, C) concentrations determined out of and during the birch pollen season (highlighted in gray) in patients with birch pollen allergy treated with SIT and after SIT (post-SIT). D, IgE/IgG ratio over the entire 54-month observation period. Median values are indicated by black lines. Discontinuities by dashed lines mark nonexistent data. Differences in pretreatment values compared with subsequent time points were analyzed by using the nonparametric Wilcoxon signed-rank test. * $P < .05$, ** $P < .01$, and *** $P < .001$.

differentiated allergen-specific T_H2 cells is the critical event in restoring tolerance during SIT rather than induction of allergen-specific Treg cells.³⁶ Because activation of allergen-specific Treg cells is influenced by an active cross-talk with T effector cells,^{40,41} the absence of Bet v 1-specific T_H cell responses

could also be responsible for the disappearance of allergen-specific T_R1 cells. Finally, by blocking allergen binding to IgE, Bet v 1-specific IgG antibodies induced by SIT can prevent IgE-FAP to T cells and thus the further propagation of Bet v 1-specific T-cell responses.⁴²

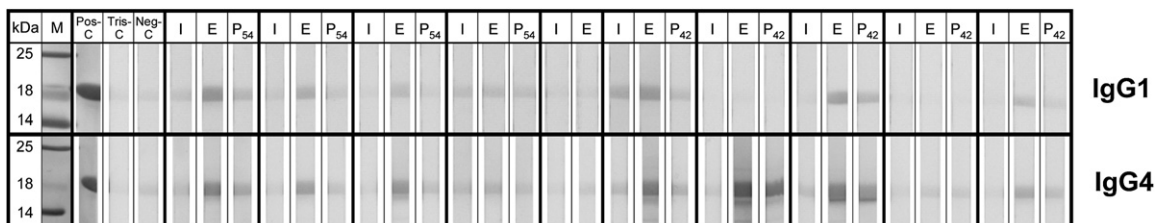


FIG 3. Bet v 1-specific IgG₁ and IgG₄ antibody levels (17 kDa) monitored in SIT-treated patients' sera by means of Western blotting at initiation of (I), end of (E), and after (P) SIT (m42 or m54 as indicated). M, Marker; Neg-C, serum of a healthy subject; Pos-C, serum of a patient with birch pollen allergy; Tris-C, buffer control.

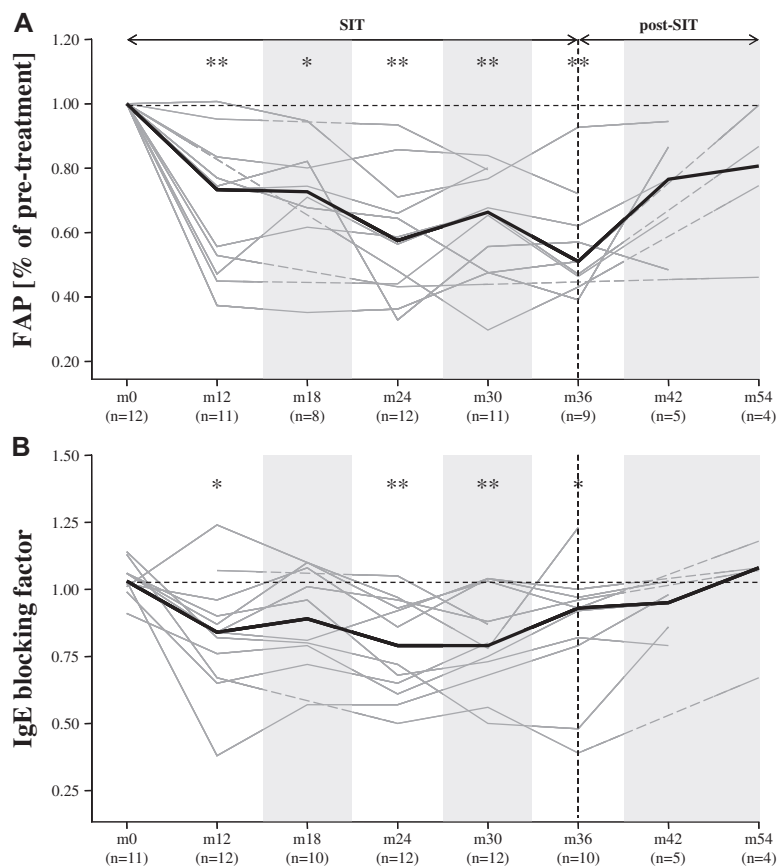


FIG 4. **A,** Analysis of allergen-IgE complex formation in SIT-treated patients with birch pollen allergy by using the FAP assay. The data from all patients is expressed as a percentage of the pretreatment value. **B,** Inhibitory capacity of treatment-induced non-IgE antibodies evaluated by using the IgE-blocking factor assay. Data are shown as a ratio of IgE binding to allergen in the presence of allergen-specific non-IgE antibodies divided by IgE binding alone. Median values are indicated by black lines. Birch pollen seasons are highlighted in gray. Discontinuities by dashed lines mark nonexistent data. Differences in pretreatment values compared with subsequent time points were analyzed by using the nonparametric Wilcoxon signed-rank test. * $P < .05$ and ** $P < .01$.

It could be speculated that the results of our immunoassays might differ when using birch pollen extract or nBet v 1 instead of rBet v 1a as the allergen. In contrast to birch pollen extract, which is a mixture of varying amounts of different allergens, Bet v 1 is a well-standardized antigen, thus enhancing the reliability of comparative studies. It shows reactivity in more than 95% of patients, with up to 60% reacting solely against it.⁴³ Even though nBet v 1 is a mixture of different isoallergens, both nBet v 1 and rBet v 1 show similar secondary structures and similar reactivity

with different patients' IgE and specific murine mAbs.⁴⁴ Although some studies have reported differences in their capacity to activate T cells,^{45,46} a more recent report showed equal stimulatory activity,⁴⁷ which we could confirm when comparing the reactivity of IL-10-producing T cells stimulated with either nBet v 1 or rBet v 1 in 3 of our patients (data not shown).

In contrast to the diminished T-cell reactivity,^{21,25,48} a constantly increasing production of allergen-specific IgG antibodies, mainly IgG₄ but also IgG₁, was observed during the 3 years of

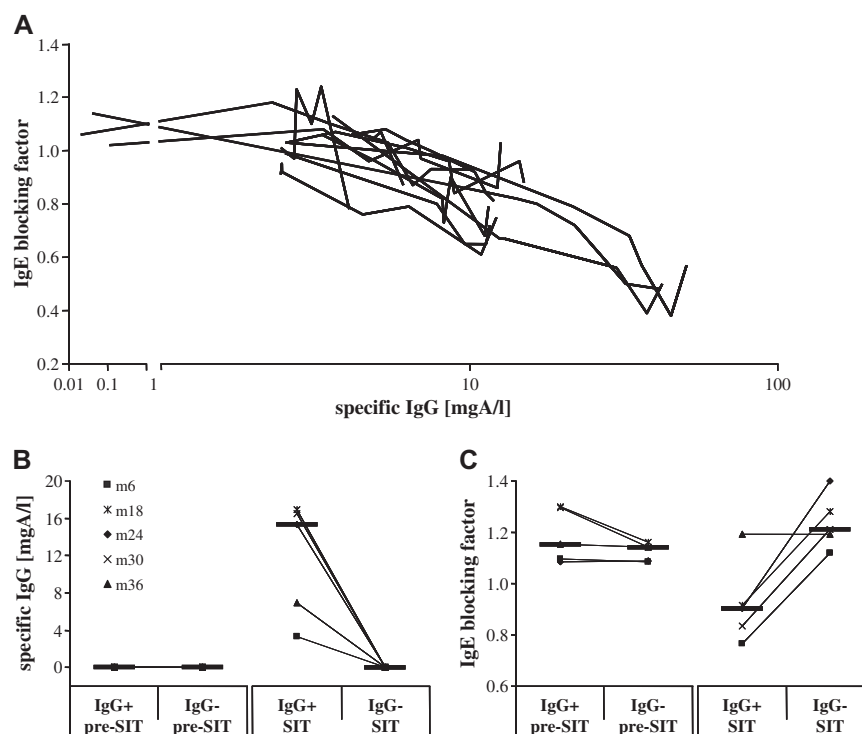


FIG 5. **A**, Inverse relationship between allergen-specific IgG antibody concentrations and IgE-blocking factor in individual SIT-treated patients for up to 54 months. **B**, Comparison of mock-treated (*IgG*+) and IgG-depleted (*IgG*-) plasma samples from 5 patients with birch pollen allergy before initiation of SIT (pre-SIT) and at different time points (months 6, 18, 24, 30, and 36) during SIT, as evaluated by using ImmunoCAP analysis. **C**, IgE-blocking factor analysis of mock-treated (*IgG*+) and IgG-depleted (*IgG*-) plasma samples of 5 SIT-treated patients before initiation of treatment (pre-SIT) and during SIT.

SIT, pointing to a T cell-independent B-cell activation in the later course of SIT. This could be due to prolonged antibody production by long-lived plasma cells^{49,50} or direct activation of allergen-specific memory B cells, which then differentiate into plasma cells.^{51,52} Although IL-10 can enhance the production of IgG₄,⁵³ this seems to be primarily important during the early induction of allergen tolerance because both the percentage of Bet v 1-specific T_H1 cells and IL-10 production by PBMCs decreased after the first year of SIT. These observations are supported by other SIT studies showing a significant increase of IL-10 secretion by PBMCs only during the first months of SIT.^{6,48,54} However, it is also possible that other IL-10-secreting cell types⁵⁵⁻⁵⁷ might substitute this Treg cell function, as has been recently shown by mouse studies demonstrating that even though the development of allergen tolerance is dependent on IL-10, it does not rely on the presence of IL-10-producing T cells (T. Jakob, personal communication).

Transient increases in allergen-specific IgE antibody levels during SIT have been reported.^{54,58} These are typically observed shortly after up-dosing. Our results show that both birch pollen and Bet v 1-specific IgE antibody concentrations largely remain unchanged in the maintenance phase and after termination of SIT, which is in line with the results of previous studies.^{21,59} This could be due to the persistence of long-lived plasma cells continuing to secrete IgE despite the loss of allergen-specific T_H2 cells, whereas the induction of allergen-blocking IgG antibodies could prevent the seasonal boost of allergen-specific IgE production.⁶⁰

Because allergen-specific IgG antibodies are able to block allergen-IgE interactions, thus inhibiting IgE-mediated antigen

presentation and histamine release,^{25,26} it has been presumed that these antibodies represent an important factor mediating allergen tolerance. *In vivo* evidence for a potential protective role of allergen-specific IgG comes from patients with bee venom allergy who became tolerant to the venom after receiving plasma IgG from beekeepers, who show increased IgG₄ responses during the bee-keeping season.⁶¹ A recent study by James et al²² suggests that the ability to block IgE-allergen binding primarily rests on the IgG₄ fraction, with inhibition being partly abolished by depletion of this isotype. Accordingly, the increase in Bet v 1-specific IgG₄ levels in our patients, which at the end of SIT constituted approximately 60% of allergen-specific IgG, was paralleled by a continuously growing blocking capacity of patients' sera, which was lost when IgG was removed. Furthermore, an inverse relationship between enhanced allergen-specific IgG₄ concentrations and decreased birch pollen SPT reactivity as a clinical parameter was noticed.

Of note, both allergen-specific IgG concentrations and the blocking capacity of patients' sera slowly decreased after discontinuation of SIT. A recent study also observed a decrease in allergen-specific IgG₁ and IgG₄ levels 2 years after finishing SIT, but no change in the inhibitory bioactivity for allergen-IgE-binding was noticed.²² It was assumed that SIT leads to affinity maturation of allergen-specific IgG antibodies, with a small, long-term, persisting antibody fraction being highly effective in inhibiting the formation of IgE-allergen complexes, and thus measuring the IgE-blocking capacity induced during SIT might be the best suited parameter correlating with the clinical outcome of SIT.²⁰ Further clinical studies extending far beyond the regular

treatment period of 3 years of SIT should help to answer this question.

One common limitation of longitudinal prospective studies in human subjects is the small number of patients participating over the whole study period, as was the case in this study. However, dropouts were not due to failure of SIT but to reasons unrelated to the study, such as moving, pregnancy, and non-compliance to adhere to the study protocol after finishing treatment. Furthermore, participants were investigated in a very closely netted study design not to miss any deviations of the analyzed immune parameters. Even when following small cohorts, which cannot be statistically analyzed, individual data lines revealed persistent alterations of T-cellular and humoral immune parameters.

In summary, we here show that SIT results in a dynamic process of both allergen-specific T-cell and humoral changes not only in the first year but also during the entire treatment period. Sustained efficacy reaching beyond the 3 years of SIT seems to depend on both loss of allergen-specific T_H2 cell reactivity and persistence of IgG antibodies with the ability to block allergen-IgE interaction. Of note, allergen-specific IgG can interfere not only with the elucidation of IgE-triggered allergic responses on effector cells but also IgE-mediated antigen presentation to T cells, which might be important for diminishing allergen-specific T_H2 cell responses. In this regard it would be interesting to gain more insight into the fate of allergen-specific T cells during SIT (ie, if loss of reactivity or shift into different compartments is a key feature for maintaining allergen tolerance) and to study the dynamics of clinical protection and allergen-specific humoral responses after finishing SIT in more detail. The lessons learned from these studies should help to improve both the efficacy and maintenance of SIT-induced allergen tolerance.

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Key message

- Allergen tolerance induced by SIT is a dynamic process involving transient induction of IL-10-secreting Treg cells, long-term loss of allergen-specific T_H2 cells, and synthesis of allergen-blocking IgG antibodies.

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METHODS

Clinical efficacy measurements

Birch pollen extract SPT reactivity was evaluated outside the birch pollen season before initiation of SIT (month 0) and every following year (months 12, 24, 36, 48, and 60), according to the guidelines of the German Society for Allergology and Clinical Immunology.^{E1} Mean wheal diameters (in millimeters) were graded semiquantitatively on a scale of 0 to 4. Skin tests results were defined as follows: 0, wheal less than 3 mm; 1, wheal of 3 to less than 4 mm; 2, wheal of 4 to less than 5 mm; 3, wheal of 5 to less than 6 mm; and 4, wheal of 6 mm or greater. Test results were exclusively evaluated by 2 specially instructed technicians to avoid interphysician variation in scoring and interpretation of SPT reactivity. Assessment of the patients' symptoms (ie, a clinical score reflecting the extent of allergic symptoms on a scale of 1 [no symptoms] to 7 [most severe symptoms]) was performed during the birch pollen season 6 months before the beginning of therapy (month -6) and over the 5-year observation period (months 6, 18, 30, 42, and 54, respectively).

Blood samples and preparation of PBMCs

Citrate-phosphate-dextrose-adenine-containing peripheral blood samples were taken from the patients at defined time points. PBMCs were isolated by using Pancoll (PAN-Biotech, Aidenbach, Germany) density gradient centrifugation, and a total of 10^7 PBMCs per milliliter were frozen in 50% (vol/vol) heat-inactivated FCS (PAA Laboratories, Pasching, Austria) and 10% dimethyl sulfoxide (Sigma, Schnellendorf, Germany). After initial gradual freezing (decrease by 1°C/min) in freezing containers (Nalgene Nunc International, Rochester, NY) to -80°C, PBMCs were kept in liquid nitrogen for long-term storage.

ELISpot assay

The ELISpot assay was performed as described previously.^{E2} Briefly, 10^6 PBMCs/mL were cultured in RPMI-1640 (Lonza, Basel, Switzerland) supplemented with 10% pooled human serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L L-glutamine (all from PAA Laboratories). PBMCs were stimulated with 10 µg/mL rBet v 1 (Biomay, Vienna, Austria) or were left unstimulated. For *in vitro* expansion of Bet v 1-specific T cells, IL-2 (10 U/mL; Roche, Mannheim, Germany) and IL-7 (10 ng/mL; Miltenyi Biotec, Bergisch Gladbach, Germany) were added after 48 hours. On day 8, PBMCs (2×10^5 /well) were restimulated with Bet v 1 (10 µg/mL) or 1% PHA (Sigma) on an ELISpot plate coated with either anti-human IFN-γ, anti-IL-5, or anti-IL-10 mAbs and were incubated for an additional 20 hours at 37°C in a humidified atmosphere containing 5% CO₂. ELISpot analysis was performed according to the manufacturer's protocol (BD Biosciences, Heidelberg, Germany). Spots were counted automatically with an ELISpot plate reader (A.EL.VIS, Hannover, Germany), and median spot numbers were determined in triplicates after subtracting the spots of unstimulated wells from the antigen-stimulated wells.

Cytokine ELISA

PBMCs (10^6 /mL) were cultured in RPMI-1640 (Lonza) supplemented with 10% pooled human serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L L-glutamine (all from PAA Laboratories) alone or in the presence of rBet v 1 (10 µg/mL, Biomay) at 37°C in a humidified atmosphere containing 5% CO₂. Supernatants were harvested after 72 hours (IL-10) and 7 days (IL-5 and IFN-γ), respectively, and frozen at -20°C until cytokine analysis by using ELISA kits according to the manufacturer's protocol (BD Biosciences). Cytokine levels were determined in duplicates and expressed as Δ values (ie, mean level in control cultures subtracted from mean level in Bet v 1-stimulated PBMCs).

Analysis of allergen-specific serum immunoglobulins by means of Western blotting

Pollen extract was isolated by using incubation of 5 g of birch pollen (Allergon, Angelholm, Sweden) per 20 mL of Tris buffer (20 mmol/L, pH 8) for 30 minutes at 37°C. Soluble extract was obtained after 30 minutes of centrifugation at 17,000g and lyophilized.

nBet v 1 was isolated from pollen extract according to the purification by Ferreira et al^{E3} with modifications. Birch pollen extract was dissolved in 0.1% trifluoroacetic acid (TFA) and run on a C8 reverse phase column (HyperClone 5µ; Phenomenex, Aschaffenburg, Germany), applying a gradient of 0% to 90% isopropanol. Further purification was performed by using gel filtration (Superdex 30; GE Healthcare, Freiburg, Germany) with 0.2 mol/L NH₄HCO₃ (pH 8).

The gene for Bet v 1a (accession no. X15877) was cloned into a pET23b vector (Novagen, Darmstadt, Germany), and rBet v 1 was expressed in *Escherichia coli*. Purification was performed by use of metal affinity chromatography, according to the manufacturer's description (Talon Superflow; Clontech, Mountain View, Calif).

SDS-PAGE was performed as described by Laemmli^{E4} on homogenous gels (T = 15%) under reducing conditions. Thirty micrograms of birch pollen extract and 5 µg of nBet v 1 or rBet v 1 (per centimeter) was separated by using SDS-PAGE. Subsequently, the proteins were transferred to nitrocellulose membrane (0.45-µm pore size; Schleicher & Schüll, Dassel, Germany) by using semidry blotting.^{E5} After blocking with 0.1 mol/L Tris-buffered saline (TBS)-TBS-T (pH 7.4 containing 0.05% Tween 20), the nitrocellulose membrane was cut into strips and incubated with patients' sera (1:20 in TBS-T). After washing, incubation was performed with alkaline phosphatase-conjugated secondary antibodies (anti-human IgE [1:10,000] and anti-human IgG₄ [1:10,000]; BD Pharmingen, Heidelberg, Germany). For detection of IgG₁, mouse anti-human IgG₁ (1:10,000; Janssen Biochemica, Amsterdam, The Netherlands) and alkaline phosphatase-labeled goat anti-mouse IgG/IgM (1:10,000; Jackson ImmunoResearch, West Grove, Pa) were used. Binding patterns were visualized by using the substrate solution NBT/BCIP (Pierce, Thermo Scientific, Schwerte, Germany).^{E6}

FAP

FAP was performed as previously described.^{E7} A stock indicator serum from a donor with birch pollen allergy containing high concentrations of allergen-specific IgE (>100 kU/mL, Advia Centaur; ALK-Abelló, Hørsholm, Denmark) was preincubated with purified Bet v 1 allergen (0, 0.3, and 1 µg/mL) at 37°C for 1 hour alone (vol-allergen:indicator:buffer = 1+1+1) or in the presence of sera (vol-allergen:indicator:SIT serum = 1+1+1) from patients with birch pollen allergy undergoing immunotherapy. Next, EBV-transformed B cells were added at 10^5 cells per sample (vol-allergen:indicator:SIT serum:B cells = 1+1+1+1), and the samples were further incubated for 1 hour at 4°C before washing and detection of surface binding of allergen-IgE complexes by using flow cytometry with a fluorescein isothiocyanate-labeled anti-IgE polyclonal antibody (F0352; DAKO, Glostrup, Denmark). All samples were analyzed in duplicates. All samples from the same patient before/after SIT were analyzed in the same experiment.

The stock indicator serum resulting in strong complex formation was used as standard serum in all FAP inhibition assays in which a rabbit anti-*Betula verrucosa* serum was used as positive inhibition control (not shown).

Measurement of serum blocking activity of allergen-IgE interaction

The IgE-blocking factor assay was performed according to the method of Würtzen et al.^{E7} In brief, all samples were tested on an Advia Centaur Instrument^{E8} by using 2 different assay procedures. The first procedure was done according to the manufacturer's instructions for specific IgE measurements in serum samples, including a washing step after catching IgE on the solid phase, removing all competitive non-IgE antibodies, before addition of biotinylated allergen (wash assay). The second procedure excluded this washing step (no wash assay), allowing non-IgE antibodies to interact with the biotinylated allergens in competition with the solid phase absorbed IgE antibodies. The read-out was the ratio between these 2 measurements (no wash/wash), which were performed as follows.

For the wash assay, the serum sample is dispensed, and solid-phase anti-IgE is added (paramagnetic particles [PMPs] covalently coupled with murine anti-human IgE antibodies). Subsequently, the PMPs are washed and resuspended in wash buffer (Bayer Diagnostics, Tarrytown, NY), followed by stepwise addition of biotinylated allergen and acridinium ester-labeled streptavidin. Finally, the

PMPs are separated from the supernatant with a magnet, the fluid phase is aspirated, and the particles are resuspended in wash buffer. This final separation procedure is repeated 3 times, and subsequently, the readout reaction is primed by the addition of hydrogen peroxide in nitric acid. The light pulse recorded by the Advia Centaur instrument (relative light units) is generated after addition of sodium hydroxide. Relative light unit responses are converted into a dose (in kilounits per liter) through an instrument standard curve.

For the no-wash assay, this procedure is performed as described above, only the washing of the PMP solid-phase serum sample mixture is omitted before the addition of biotinylated allergen. The response is recorded as relative light units and can be converted to a dose (in kilounits per liter) by using the instrument standard curve. The ratio between the readout from the 2 assay procedures is a measure of the interference/inhibition by non-IgE antibodies on the binding of IgE to the allergens. All samples were analyzed in duplicate. All samples from the same patient before and after immunotherapy were analyzed in the same experiment.

Depletion of IgG antibodies

Plasma was collected from patients showing high allergen-specific IgG concentrations during SIT and compared with patients' samples drawn before SIT. All samples were incubated either with protein G-Sepharose beads (Bio Vision Research Products, Mountain View, Calif) or Sepharose beads (Sigma-Aldrich Chemie GmbH, Schnellendorf, Germany) as a mock control for 4 hours at room temperature. Afterward, sera were centrifuged and incubated with fresh beads overnight at 4°C.^{E9} These steps were repeated until complete depletion of allergen-specific IgG and IgG₄ was demonstrated by using ImmunoCAP analysis.

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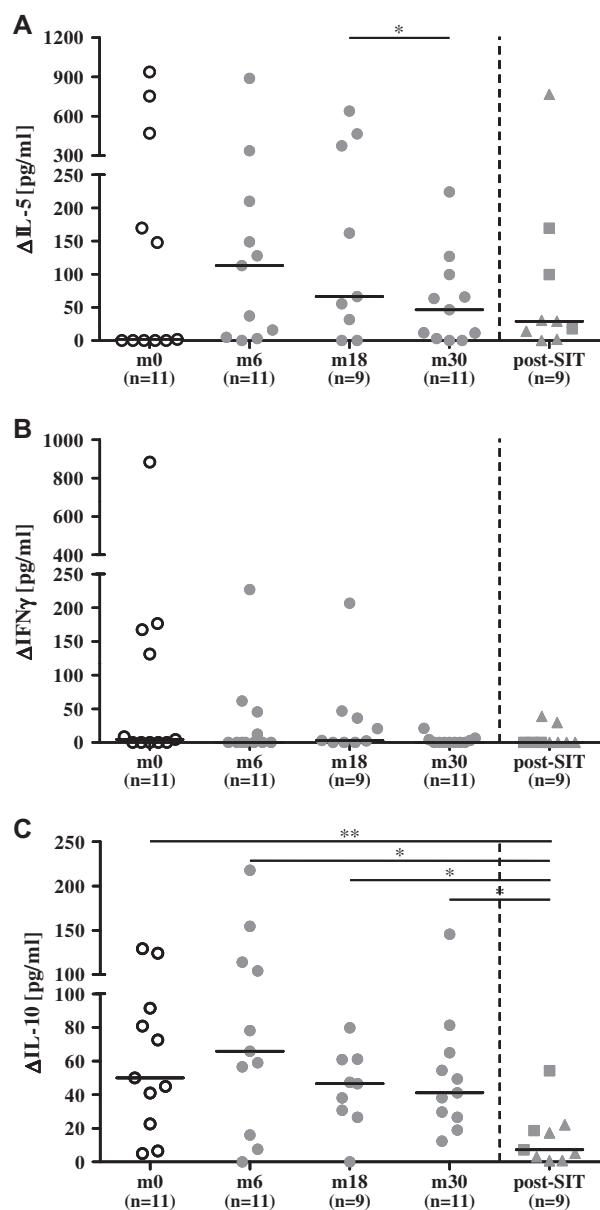


FIG E1. Specific IL-5 (**A**), IFN- γ (**B**), and IL-10 (**C**) production by PBMCs stimulated with rBet v 1. Cytokine levels were measured during 30 months (circles; m0-m30) of specific SIT and a post-SIT phase showing individual results at the last visit after termination of treatment (squares, month 42; triangles, month 54). Median values are indicated by black lines. Birch pollen seasons are highlighted in gray. Differences between initiation of SIT and during/after SIT were analyzed by using the nonparametric Wilcoxon signed-rank test. * $P < .05$ and ** $P < .01$.

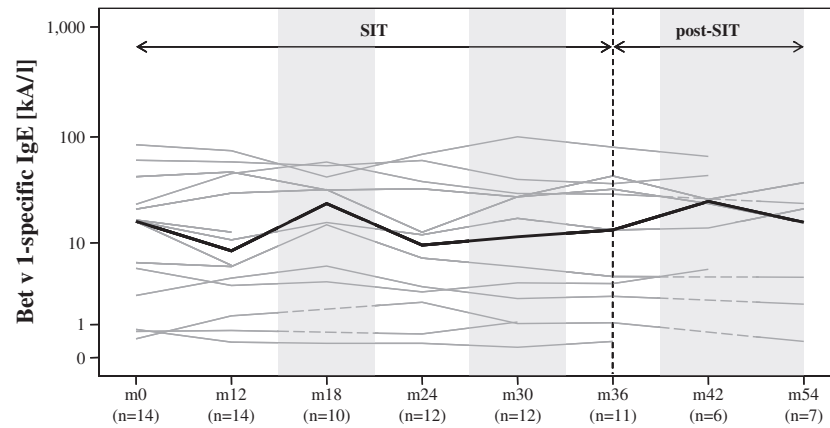


FIG E2. Bet v 1-specific IgE antibody concentrations determined out of and during the birch pollen season (highlighted in gray) in patients with birch pollen allergy treated with SIT and after cessation of treatment (post-SIT). Median values are indicated by *black lines*. Discontinuities by *dashed lines* mark nonexistent data.

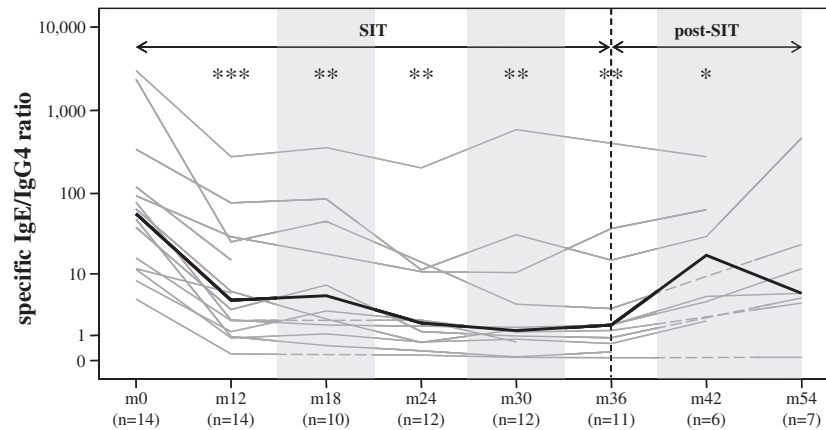


FIG E3. Birch pollen-specific IgE/IgG₄ ratios determined out of and during the birch pollen season (highlighted in *gray*) in patients with birch pollen allergy treated with SIT and after cessation of treatment (post-SIT). Median values are indicated by *black lines*. Discontinuities by *dashed lines* mark nonexistent data. Differences in pretreatment values compared with subsequent time points were analyzed by using the nonparametric Wilcoxon signed-rank test. * $P < .05$, ** $P < .01$, and *** $P < .001$.

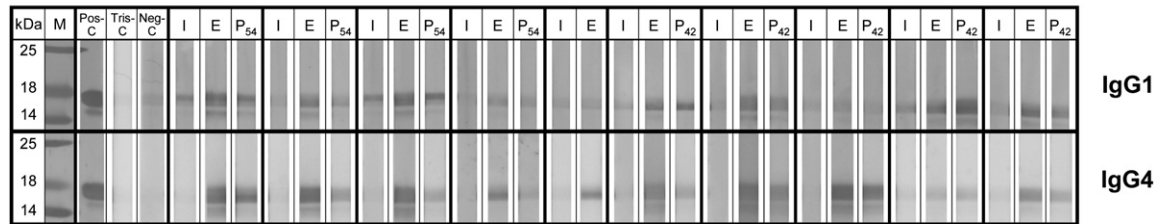
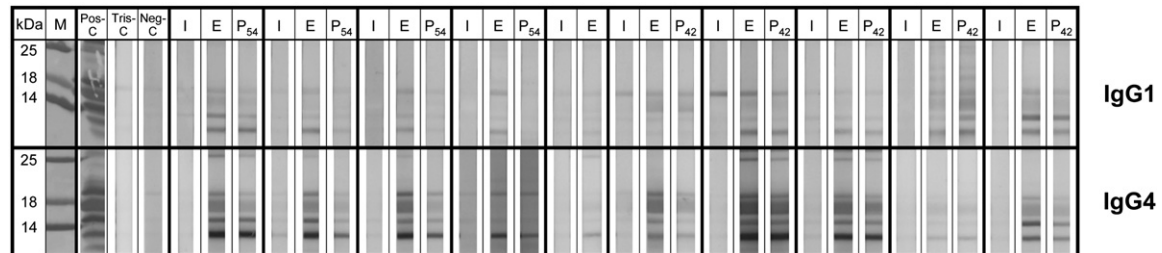
A natural Bet v 1**B** birch pollen extract

FIG E4. nBet v 1-specific (**A**) and birch pollen extract-specific IgG₁ and IgG₄ antibody levels (**B**) monitored in SIT-treated patients' sera by means of Western blotting. Concomitant alterations in IgG₁ and IgG₄ levels were detectable comparing initiation of (*I*), end of (*E*), and time points after (*P*) SIT (month 42 or 54, as indicated). *M*, Marker; *Neg-C*, serum of a healthy subject; *Pos-C*, serum of a patient with birch pollen allergy; *Tris-C*, buffer control.