

Is Immunotherapy-Induced Birch-Pollen-Specific IgG4 a Marker for Decreased Allergen-Specific Sensitivity?

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Key Words

Allergy · Birch · Clinical endpoints · IgE · IgG · IgG4 · Immunotherapy · Prognosis

Abstract

Background: The role of IgG4 during allergen-specific immunotherapy (SIT) is still controversial. The available studies present paramount differences in in vitro techniques, allergens, and clinical outcome parameters. By implementing a sensitive method, and pivotal clinical outcome parameters, we wanted to ascertain the utility of IgG4 as a clinical marker of decreased allergen-specific sensitivity to a common aeroallergen. **Methods:** Sera were drawn from 23 birch-pollen-allergic patients during a placebo-controlled clinical trial on birch pollen SIT. Seventeen patients received active treatment. Blood samples were drawn at 0, 2, 4, 7, and 30 treatment weeks, and 36 months. The binding activity of autologous IgG, IgG4, IgE, and IgE- and/or IgG-depleted serum to ¹²⁵I-labelled recombinant Bet v 1 was assessed in a fluid-phase radioimmunoassay. Disease severity was assessed subjectively on a visual analogue scale (VAS), and objectively by intradermal late-phase reaction diameters. **Results:** Before SIT IgG4 fraction of IgG-allergen binding varied from 4 to 74%, with a median of 36%,

increasing to 71% after 36 months. Changes in IgG4 or IgG4/IgG fraction were not correlated to clinical outcome parameters. Changes in IgG allergen binding and VAS were significantly correlated ($\sigma = 0.72$; $p < 0.05$). SIT increased the serum-blocking activity of IgE allergen binding from 25% before SIT to 80% after SIT. No changes were observed in the placebo group. **Conclusion:** The data suggest that IgG4 per se is a poor marker of decreased allergen-specific sensitivity to birch pollen, both as a single measurement and as delta values.

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Introduction

Recent research on bee venom and house dust mite (Der p) allergy has demonstrated that allergen-specific immunotherapy (SIT) induces IL-10 synthesis leading to T cell anergy, increased IgG4 levels, and decreased allergen-specific sensitivity [1, 2]. It was concluded that the well-known IgG4-dominated humoral response (subsequently referred to as 'IgG4 polarization') during SIT is a marker of induced decreased allergen-specific sensitivity [1–3]. Many authors have investigated the relationship between IgG and its subclasses to SIT outcome, and the results are contradicting: IgG4 increases have been associ-

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ated with clinical efficacy [2, 4–9] as well as no efficacy [10–12] of SIT. Differences in in vivo techniques for IgG and IgG subclass measurements in allergens and in clinical outcome criteria might be responsible for the diverse findings among the studies. Before SIT, pronounced differences in IgG4 levels were observed in both a bee venom and a Der p study [1, 2], suggesting that IgG4 polarization per se is a poor marker of decreased allergen-specific sensitivity. However, IgG4 polarization could be due to ongoing allergen exposure that stimulates IgG4 synthesis [13]. Thus, we aimed at optimizing the in vitro techniques and clinical settings by measuring the allergen binding activity of IgG and IgG4 in the same sensitive fluid-phase assay [14] in IgE-depleted sera from patients undergoing SIT to birch pollen [15]. Furthermore, the SIT-induced changes in IgG and IgG4 were subsequently correlated to pivotal clinical SIT study outcome parameters: the visual analogue scale (VAS) for subjective improvement, and the intradermal late-phase reaction for objective assessment [3, 15, 17].

Patients and Methods

Patients and Serum Samples

Blood samples were drawn at 0 (January 2000), 2, 4, 9, and 30 weeks in 23 birch-pollen-allergic patients (active, $n = 17$; placebo, $n = 6$) participating in a double-blinded and placebo-controlled clinical study on birch pollen SIT [15]. No patient had ever received SIT before. An additional blood sample was drawn at 36 months in 11 of the SIT-treated subjects, selected on the basis of a high Bet v 1 IgE binding. Ten patients subjectively assessed the severity of symptoms on a VAS at 0 and 30 weeks, and at 36 months (1 patient had falsely low VAS at inclusion due to earlier treatment with systemic corticosteroids). The objective endpoint, the intradermal late-phase reaction to birch pollen, was performed at 0 and 30 weeks in 9 patients since 2 were pregnant. Both endpoints decreased significantly during SIT [15]. All patients suffered from allergic symptoms at week 7 (birch pollen season), and none at any other blood sample collection. SIT was performed according to international guidelines and a safe dose-increasing regimen [3, 15]. The study was approved by the local ethics committee.

CAP Measurement of IgE to Bet v and Recombinant Bet v 1

Bet v 1 is the major allergen in birch [18], and recombinant forms have been produced. To ascertain the biological relevance of the recombinant isoform used in the present study, rBet v 1.2801 (expressed in *Escherichia coli* [19]; ALK-Abellø, Hørsholm, Denmark), we performed a CAP inhibition assay. Specific serum IgE (SIT-treated patients; $n = 17$) was measured in duplicate using the CAP system (Pharmacia, Uppsala, Sweden). CAP inhibition was performed in duplicate: 50% serum was pre-incubated at 4°C for 18 h with one of the two competitors: native birch extract Bet v (Aquagen, ALK-Abellø), 10 or 50 µg/ml, or rBet v 1, 1 or 5 µg/ml. In preliminary studies, maximal inhibition by Bet v was observed at 50 µg/ml (however, only marginally different from that at 10 µg/ml). The residual

Table 1. Inhibition by the isoform Bet v 1.2801 of birch pollen (Bet v)-specific IgE measured in the CAP analysis system (median and range inhibition (Bet v, 50 µg/l); of non-inhibited CAP analyses (%) $n = 16$)

Duration of SIT	rBet v 1, 1 µg/l	rBet v 1, 5 µg/l	Bet v, 10 µg/l
0 weeks	97.4 (81.8–100)	100 (74.8–100)	97.1 (92.1–100)
30 weeks	94.8 (80.2–100)	96.0 (81.5–100)	96.6 (84.5–100)
p	>0.1*	<0.05*	<0.05*

* Wilcoxon's test for paired comparisons.

activity did not differ between 0 and 30 weeks at any concentration ($p > 0.15$), and was, as background value, subtracted from rBet v 1 CAP inhibition results presented in table 1. Isotonic saline served as diluent and buffer control.

Assays for Bet v 1 Binding to Serum IgE and IgG

In a previous work, we found the CAP system to be less useful in detecting specific IgG reactivity because of high non-saturable binding values. Also, the high concentration of allergen in this assay system makes the detection of specific IgE insensitive to blocking IgG in the sample. In contrast, this was not observed when testing with ^{125}I -labelled allergen [14]. Therefore, to compare the binding of Bet v 1 to serum IgE and IgG, separated or together, we used Bet v 1.2801 (molecular weight: 17 kDa) that was labelled with ^{125}I using chloramine-T and followed by molecular size chromatography on a Sephadex G-75 superfine column (Pharmacia) [20]. More than 90% of the purified ^{125}I -Bet v 1 bound to specific IgE and IgG from birch-pollen-allergic patients. The specific activity was $1\text{--}3 \times 10^5$ cpm/ng.

All sample dilutions were made in assay buffer (0.02 M phosphate buffer, 0.125 M NaCl, pH 7.4, 0.025% gelatin (type A from porcine skin; Sigma, St. Louis, Mo.) and 0.1% Triton X-100 (Sigma)), and binding tests were performed in duplicate after 18 h of pre-incubation at 4°C with ^{125}I -Bet v 1. The results were corrected for non-saturable, nonspecific binding in the presence of 200–500 ng/ml of unlabelled Bet v 1. The nonspecific binding was generally below 2%. Determinations of specific activity were done with errors below 2% in a γ -counter (Wizard 1470 automatic gamma counter, Wallac OY, Finland).

Binding to IgG

Before specific serum IgG determinations (all patients, $n = 23$), serum was depleted of IgE by co-incubation with a supraoptimal amount of anti-IgE monoclonal antibody coupled to paramagnetic beads (ALK-Abellø) for 5 h at 4°C [21]. Complete IgE removal was ascertained by co-incubating the IgE-depleted serum samples with anti-IgE beads followed by tests for Bet v 1 binding to IgE as described later.

^{125}I -Bet v 1 binding to IgG, including all the IgG subclasses, was determined by affinity separation on columns containing 0.5 ml of protein G Sepharose CL-4B. 150 µl of sample containing a maximum of 10% IgE-depleted serum were applied. Non-bound ^{125}I -Bet v 1 was eluted in 3 ml of assay buffer, and the ^{125}I -Bet v 1 bound to IgG as well as IgG was eluted with 3 ml of 0.1 M glycine-HCl, pH 2.4.

Radioactivity in both fractions was determined for estimation of the ratio between bound and free Bet v 1 binding activity. This ratio was compared to an internal standard IgG preparation where the binding activity of 1 mg/ml was assigned the arbitrary value of 1 U/ml.

Binding to IgG4

The relative contribution of IgG4 to IgG-Bet v 1 complexes was estimated by simultaneous anti-IgG4 and protein G affinity separation of IgE-depleted serum pre-incubated with ^{125}I -Bet v 1 in the 11 patients followed for 36 treatment months.

The anti-IgG4 affinity columns were made in the following way. First, dialyzed mouse anti-human IgG4 monoclonal antibody G4.T9 (ALK-Abellø) was immobilized on divinyl sulfon agarose beads (Mini-Leak Low; Kem-En-Tec, Copenhagen, Denmark) according to the manufacturer's instructions using 2.5 mg IgG per gram of agarose. Each column contained 1 ml of anti-IgG4 antibody-agarose beads [22]. Next, the specificity of the affinity column with respect to IgG4 was confirmed by ELISA for IgG1 and IgG4 (at weeks 0 and 30) in equally diluted IgG4-depleted and non-depleted serum samples using anti-human IgG1 and IgG4 (The Binding Site, Birmingham, UK) as catching antibodies (dilution 1:1,000) and horseradish-peroxidase-coupled sheep anti-human IgG1 and IgG4 (The Binding Site; dilution 1:1,000 and 1:500, respectively) as secondary antibodies. In IgG4-depleted samples, we found 9% (range 3.6–24%) of IgG4 but 99% (range 77–140) of IgG1 compared to non-depleted serum samples, without significant differences between weeks 0 and 30 (data not shown).

Measurements were made at a concentration of IgG binding 50% of total ^{125}I -Bet v 1. Simultaneously, 100–200 μl of the same sample were added to the anti-IgG4 and the protein G column. Non-bound and IgG-bound ^{125}I -Bet v 1 was eluted as described above for the protein G Sepharose CL-4B columns. For all samples, the unspecific binding on both anti-IgG4 and the protein G columns was determined following co-incubation of the sample with ^{125}I -Bet v 1 together with 200 ng/ml of Bet v 1 and using the same columns as when testing the sample co-incubated with ^{125}I -Bet v 1 alone. The nonspecific binding varied between 2 and 7% for the anti-IgG4 column and between 2 and 5% for the protein G column.

The reproducibility of the measurements was from 76 to 99% and quantitation of Bet v 1 binding to IgG4 was made as described above for the binding to IgG.

Binding to IgE

IgE anti-Bet v 1 was detected by the binding of ^{125}I -Bet v 1 to IgE absorbed to anti-IgE-antibody-coated paramagnetic beads. To test blocking activity in the serum, 25 μl of ^{125}I -Bet v 1 were at the same time added directly to 150 μl of 20% serum pre-incubated with the beads for 3 h and to the pre-incubated sample following two washings of the beads with 1 ml of assay buffer and reconstitution to the original sample volume. After co-incubation for 18 h at 4°C, free and washing the beads 2 times with 1 ml assay buffer separated bound ^{125}I -Bet v 1. The background cpm bound to the beads was always less than 1% of added cpm.

Statistics

Data were analyzed with statistical computer software (SPSS for Windows, 10.0.7), and continuous data with the Mann-Whitney test, paired continuous data with paired Wilcoxon's U test, and correlations with Spearman's rank correlation test.

Results

IgE Reactivity to rBet v 1.2801

Serum levels of birch-pollen-specific IgE (CAP system) were measured before SIT (0 weeks) and after 2 years. The initial results were reproduced in all but one serum sample, which was omitted from the present analysis. Specific IgE levels increased insignificantly from a median of 42.2 to 46.9 $\text{kU}_\text{A}/\text{l}$ ($n = 16$) at 30 treatment weeks, and to 58.9 $\text{kU}_\text{A}/\text{l}$ ($n = 11$) after 36 months of treatment. Maximal inhibition with native allergen (Bet v at 50 $\mu\text{g}/\text{ml}$) of the CAP analysis resulted in a residual activity of 0.65 (range 0.35–2.79) $\text{kU}_\text{A}/\text{l}$, corresponding to a reduction of more than 95% of the non-inhibited CAP result. We subtracted this background signal from the IgE measurements when CAP analysis was inhibited with recombinant allergen (1 or 5 $\mu\text{g}/\text{ml}$), or native allergen (10 $\mu\text{g}/\text{ml}$). Table 1 shows that recombinant allergen, rBet v 1.2801, inhibited the CAP analysis >94%, and that CAP inhibition decreased from 0 to 30 treatment weeks. We observed no significant differences between recombinant or native allergen forms at either 0 nor 30 treatment weeks (all p values >0.3; Wilcoxon's test for paired comparison; test not performed at 36 months). CAP inhibition results divided the patients into two distinct groups: 12 subjects had more than 95% CAP inhibition by rBet v 1, 5 $\mu\text{g}/\text{ml}$ (median 100, range 96.5–100%), whereas 4 patients had less than 95% CAP inhibition (median 90.9, 74.8–94.5%).

Bet v 1 Binding to IgG and IgG4 in IgE-Depleted Serum

Figure 1 illustrates that SIT increased the level of specific IgG-Bet v 1 binding seven-fold, predominantly within the 1st year of treatment ($n = 11$; from weeks 2 to 30; significant visit-to-visit increase, $p < 0.05$; $n = 17$; fig. 3). Figure 2 shows that the IgG4 fraction of IgG-Bet v 1 complexes increased continuously ($n = 11$), showing decreasing interindividual variability.

Bet v 1 Binding to Serum IgE in the Absence and Presence of Serum

SIT subjects experienced a significant and continuous increase in IgE-Bet v 1 binding until week 7 in the absence of serum. With serum present, IgE-Bet v 1 binding decreased steadily (significant from week 4 on), reaching 38% of the pre-treatment levels at week 30 (fig. 3). Furthermore, serum significantly reduced IgE-Bet v 1 binding, being 25% before treatment (week 0) and 80% at 30 weeks (fig. 3).

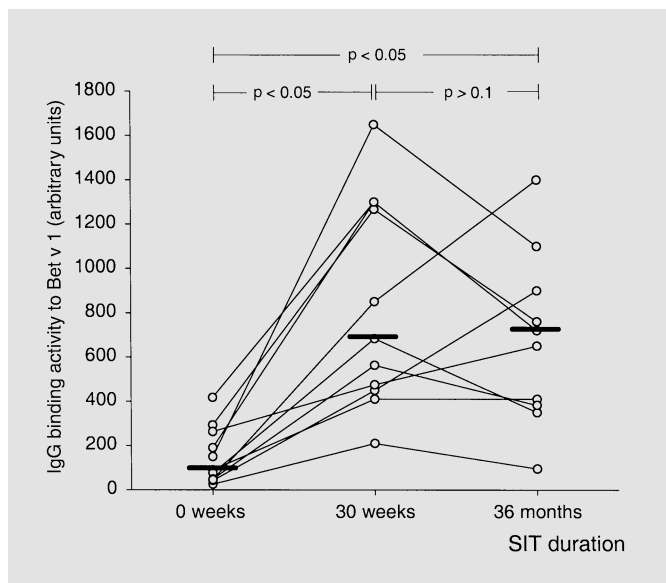


Fig. 1. IgG reactivity to Bet v 1 before and during SIT (bars: median values). IgE-depleted sera were incubated with ^{125}I -labelled Bet v 1.2801. Binding was assessed by protein G absorption and expressed in arbitrary units (see text for definition). Data are shown as mean values of duplicate experiments. Each line represents 1 patient. Wilcoxon's paired rank sum test.

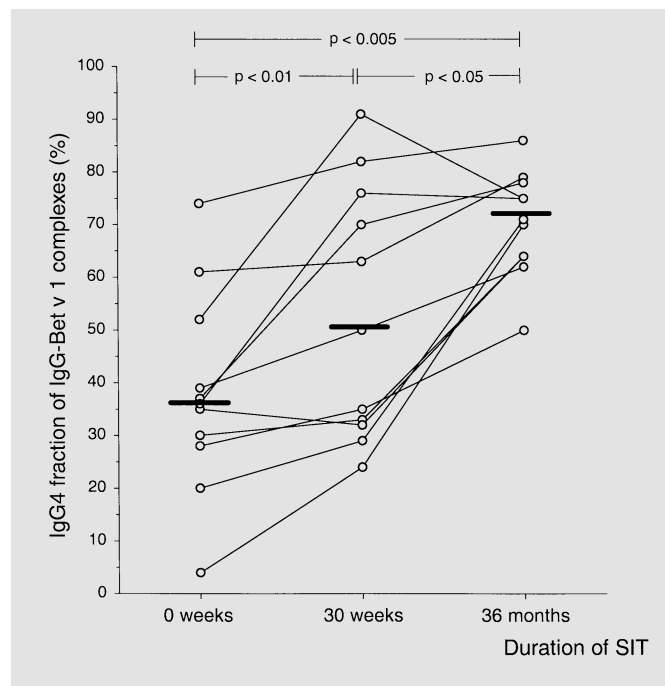


Fig. 2. The relative contribution of IgG4 to IgG-Bet v 1 complexes before and during SIT (bars: median values). IgE-depleted sera were incubated with ^{125}I -labelled Bet v 1.2801. Binding was assessed by absorption to protein G and anti-IgG4 columns and expressed in arbitrary units (see text for definition). The difference in binding to protein G and anti-IgG4 columns was attributed to IgG4. Data are shown as mean values of duplicate experiments. Each line represents 1 patient. p values are based on Wilcoxon's paired rank sum test.

Fig. 3. IgE and IgG binding to Bet v 1, and blocking of IgE binding to Bet v 1 by serum at several time points during SIT (treatment weeks 0, 2, 4, 7, and 30; $n = 17$). IgE-Bet v 1 binding was expressed in relation to binding at week 0 in the absence of serum. IgG binding was expressed in relation to IgG binding at 0 weeks. IgE binding to Bet v 1 was measured by adsorption to anti-IgE-coated paramagnetic beads followed by incubation with ^{125}I -labelled rBet v 1.2801 with (closed circles; serum absent) or without serum (open circles; serum present) prior washing. IgG binding to Bet v 1 (open triangles) was measured in IgE-depleted sera incubated with ^{125}I -labelled rBet v 1.2801, and subsequent protein G separation. A bound/total (B/T) value was made for each sample: bound ^{125}I -labelled rBet v 1.2801 (cpm) divided by the total activity (cpm). The mean of duplicate tests was used for calculation, and data are shown as median values with ranges. See text for statistical evaluation of differences.

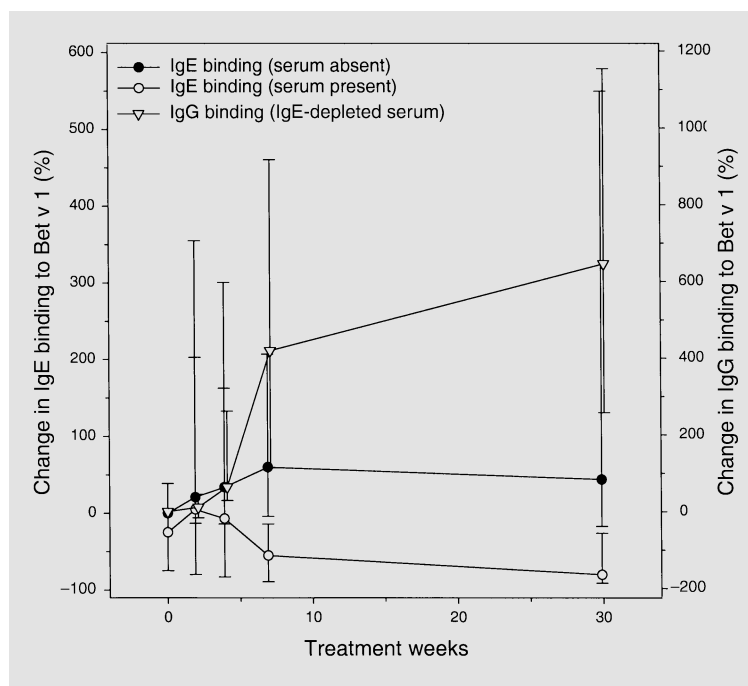


Table 2. Correlation of SIT-induced changes in clinical endpoints and changes in Bet v 1 binding to specific IgG4, IgG, and IgG4/IgG after 30 weeks of SIT (correlation coefficient, σ)

	IgE in serum (n = 17)	IgE no serum (n = 17)	IgG4 (n = 11)	IgG (n = 11)	IgG4/IgG (n = 11)
VAS	0.12	0.36	0.48	0.72*	-0.18
LPR	0.42	0.21	-0.58	-0.50	-0.23

Change in clinical outcome parameters: value at 30 weeks divided by value at 0 weeks. LPR = Intracutaneous late-phase reaction. * $p < 0.05$ (Spearman's rank correlation test).

In placebo-treated subjects, the presence of serum significantly reduced IgE-Bet v 1 binding at all times ($p < 0.03$), with no change over time (median: -11, range -36 to -27%; $p > 0.7$, data not shown). At 30 weeks, IgE-Bet v 1 binding increased significantly, with a median of 47% (range 7-64%; $n = 6$; $p < 0.05$), regardless of the presence or absence of serum ($p > 0.7$, data not shown).

Immunoglobulins and Clinical Endpoints

The relative reduction in subjective symptom severity assessed by VAS scoring at 30 weeks was positively and significantly correlated to the change in IgG – but not IgG4 – binding to Bet v 1 (fig. 4). Table 2 shows that no other significant correlation between clinical and serologic variables was present after 30 weeks of treatment. Likewise, we found no significant correlation after 3 years of treatment (all p values > 0.4 ; $n = 11$; data not shown).

Discussion

This study challenges the view that specific IgG4 is a useful clinical marker of decreased allergen-specific sensitivity [1, 2, 7, 9]. Even though our results followed the well-known SIT study pattern of increased IgG levels with a preferential increase in IgG4 [3], we confirmed the presence of high interindividual variability in IgG4 polarization [1, 2]: post-SIT IgG4 polarization was in several patients lower than the pre-SIT values in other patients (fig. 2). Most importantly, we found no correlation between SIT-induced changes in IgG4 fraction to either subjective nor objective clinical data (table 2). Our data support the assumption that IgG4 polarization (1) per se does not imply decreased allergen-specific sensitivity [13], and

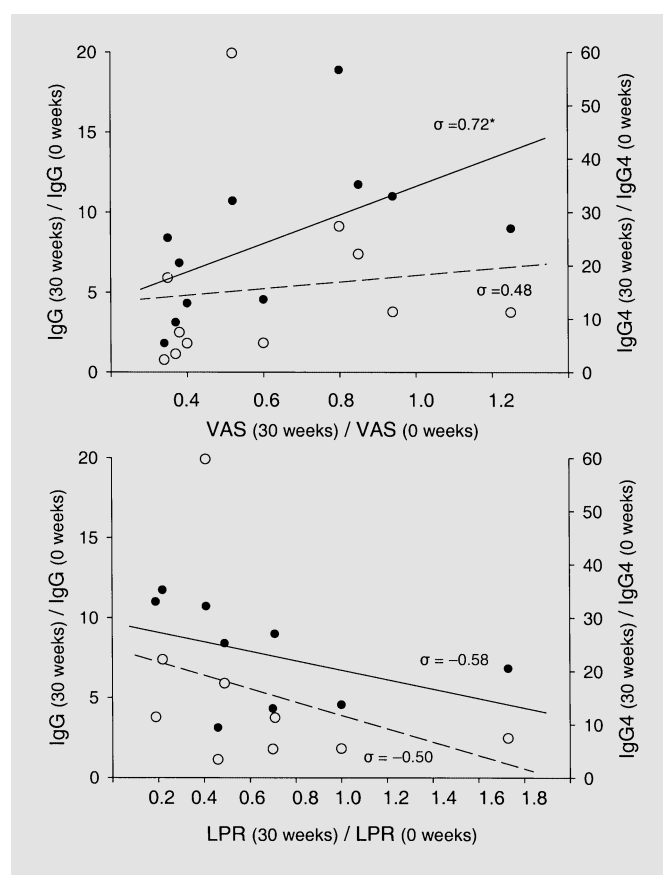


Fig. 4. The correlation of the relative increase in Bet v 1 binding activity by IgG (closed circles, solid line) and IgG4 (open circles, dashed line) to the relative change in VAS and cutaneous late-phase reactions (LPR). Relative change: values at 30 treatment weeks divided by values at 0 treatment weeks. * $p < 0.05$; Spearman's rank correlation test.

(2) is a poor marker of SIT efficacy [23]. The choice of a seasonal allergen made it unlikely that IgG4 levels measured out of season were affected by allergen exposure [13, 24], since at least 3 months elapsed between season termination and blood sample collection. The clinical outcome could have been affected by the presence of other allergies, or an insufficient treatment. However, no patient presented or developed any allergy resembling birch pollen symptoms, and the clinical study was efficacious, and performed according to international guidelines [3, 15]. The clinical outcome parameters are those most affected by SIT, and they are frequently used in SIT studies [3, 17]. Our findings are strengthened by the fact that IgG and IgG4 (i.e. IgG-IgG₁₋₃) binding activities were measured in the same assay, which eliminated interassay differ-

ences. Most measurements of different immunoglobulins use different ELISAs or other solid-phase assays [1, 2, 6, 7, 16, 25, 26]. In the same assay, we could show that the SIT-induced IgG was of suitable affinity to inhibit IgE allergen binding (fig. 3). This 'blocking' capacity was present in untreated subjects, which is in accordance with another study using a fluid-phase assay but not studies using solid-phase assays [6, 26]. 'Blocking' activity increased during SIT, whereas the placebo-treated group only experienced an increase in IgE allergen binding after 30 treatment weeks [27], corresponding to seasonal boosting of serum IgE synthesis [28]. Altogether, using anti-IgE-antibody-coated paramagnetic beads and radiolabelled allergen proved to be a sensitive method for measuring immunoglobulin binding capacity [14, 21].

We have previously shown that IgG4 depletion resulted in equivalent reductions in binding and blocking activities of Bet v 1 to IgE [29]. We have not been able to find significant alterations in IgG or IgG4 avidity by aeroallergen SIT [14, 29], which have been shown in insect venom studies [30, 31]. This supports that IgG4 contributes to the blocking activity by simple quantity rather than altered quality [32, 33]. Evidently, different handling of allergen in skin and respiratory mucosa, and of seasonal and perennial allergens, accounts at least partially for the observed differences among studies [1, 34]. Our data suggest that both IgG and IgG4 are poor markers of decreased allergen-specific sensitivity.

We found that the isoform Bet v 1.2801 was as capable of inhibiting birch-pollen-specific IgE measurement as

was native extract, Bet v (table 1). We found that 75% of the patients had an almost complete inhibition by Bet v 1.2801, whereas 25% had an incomplete (75–95%) inhibition. This is analogous to earlier studies on Bet v 1, and is considered to be due to sensitization to minor allergens, predominantly Bet v 2 (profilin) [18, 35]. CAP inhibition decreased during the study (table 1), supporting that SIT induces sensitization to determinants not recognized prior to treatment [25, 36]. The clinical impact is unknown but is probably modest as the decrease in CAP inhibition is limited (table 1). Overall, Bet v 1.2801 appears to possess sufficient and relevant epitopes recognized by preformed antibodies to substitute native Bet v and Bet v 1 for in vitro use.

In conclusion, IgG4 polarization was not a clinically useful marker of either decreased allergen-specific sensitivity or of SIT efficacy due to huge interindividual variability and no correlation to clinical outcome. Furthermore, Bet v 1.2801 proved to be a relevant substitute of both Bet v and Bet v 1 for in vitro use. A future study could focus on the possible role of IgG and its subclasses in asymptomatic, aeroallergen-sensitized subjects.

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