

Vaccination for birch pollen allergy: comparison of the affinities of specific immunoglobulins E, G1 and G4 measured by surface plasmon resonance

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Summary

Background Allergen-specific immunotherapy (SIT) is associated with increased levels of allergen-specific IgG in serum. However, it is not clear to what extent qualitative changes in the allergen binding capacity of IgG may be induced as well.

Objective The purpose of this study was to investigate the influences of SIT on antibody affinity.

Methods The binding affinity of purified serum IgG1, IgG4 and IgE to the major allergen in birch (*Betula verrucosa*) pollen, Bet v 1, was analysed by surface plasmon resonance. The antibodies were obtained from 10 birch pollen-allergic patients receiving SIT and from 10 patients with no SIT.

Results The patients having received SIT have a significant higher titre of anti-Bet v 1 antibodies in their blood, but the affinity to Bet v 1 of allergen-specific IgE, IgG1 and IgG4 does not differ between the two groups. For IgG1 and IgG4, correlations between less allergic symptoms and affinity of the antibodies were observed both in the SIT group and to a smaller extent in the non-SIT group.

Conclusion SIT has no effect on antibody affinity of allergen-specific IgE, IgG1 or IgG4. Allergic patients with high-affinity IgG1 and IgG4 antibodies report less symptoms than patients with low-affinity antibodies.

Keywords affinity, allergen specific immunotherapy, allergy, Bet v 1, IgE, IgG1, IgG4, surface plasmon resonance

Submitted 19 December 2003; revised 7 September 2004; accepted 23 November 2004

Introduction

The symptoms of immediate- and late-type allergic reactions can be ameliorated by various pharmacologic treatments, but yet the only disease-modifying approach for the IgE-dependent allergy is allergen-specific immunotherapy (SIT), which was first introduced in 1911 [1] (for recent reviews, see [2, 3]).

The effects of SIT are well characterized in terms of reduced symptoms and medication scores, but the underlying biomolecular processes are less well understood. SIT seems to have little effect on the amount of allergen-specific IgE in serum. An increased production of other allergen-specific antibodies is observed after SIT [4, 5]. These 'blocking antibodies' belong to the IgG isotype, and especially a rise in allergen-blocking IgG of the IgG4 class is observed [6, 7]. This can be explained by recent studies suggesting a shift from the T-helper type 2 (Th2) cytokines IL-4 and IL-5 produced from CD4⁺ T cells to Th1 cytokines (e.g. IFN- γ and IL-10) [8] in successful SIT. Later clinical improvement is associated with a reduction in allergen-specific IL-4 production and in allergen-specific serum IgE [9, 10]. In general, a successful SIT appears to be associated with a rise in allergen-specific IgG4,

the generation of IgE-modulating CD4⁺ T cells and a reduction in the number of mast cells and eosinophils, and their release of mediators [11–13]. However, the changes in antibody responses cannot be fully explained by either the amount of these cytokines or their ratio, as it has been demonstrated that both IgG4 and IgE production is dependent on Th2 cytokines such as IL-4 and IL-13 and is down-regulated by IFN- γ [14–16].

It has been shown that there are differences in the specificity of IgG antibodies from non-allergic and allergic individuals and the epitopes that are recognized by these antibodies. Some investigators suggest that all persons in an exposed population have IgG antibodies recognizing allergens but patients allergic to a certain allergen have antibodies that recognize other epitopes on the certain allergen than non-allergic persons [17, 18]. However, this has not been shown for birch allergens. When patients become asymptomatic after SIT, the antibody specificity seems to return to that expressed by non-allergic individuals [18, 19].

It has previously been suggested that SIT changes the balance between allergen-specific IgE and IgG not only regarding the amount/titre but also with regard to affinity [20]. However, recently, it has been observed that SIT induced inhibition of the binding between allergen-specific IgE and the major birch pollen allergen Bet v 1 can be explained mainly or solely by induction of increased amounts of specific IgG [21].

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Methods

Patients

Sera were collected from two groups of patients allergic to birch pollen. One group included 10 patients who had not received SIT (seven men, three women); the second group included 10 patients having received treatment with SIT for at least 1.5 year (six men, four women). The SIT procedure is described in detail in [22]. All blood samples were collected in the birch pollen season 2002. The median age of the two groups was 29 years (range 25–34) and 27 years (range 24–48), respectively. The median duration of birch pollen-allergic rhinoconjunctivitis was 7 years (range 2–20) and 9 years (range 4–25) in the two groups, respectively. All had positive skin prick tests (SPT) to *Betula verrucosa* (Soluprick SQ, ALK-Abelló, Hørsholm, Denmark) with a weal diameter greater than 3 mm, and a positive serum test for birch pollen-specific IgE of class 3 or greater (Pharmacia CAP System, Pharmacia Diagnostics, Uppsala, Sweden). All patients assessed the allergy severity (hayfever symptoms experienced during the local pollen season) with a mark on a visual analogue scale (VAS): a 100 mm line with 0 mm representing no symptoms, and 100 mm representing the worst possible symptoms [22]. There were no statistical differences between the two groups in relation to gender, age and the duration of their birch pollen allergy. All SIT-treated subjects reported a positive effect of SIT, except one patient, who reported unchanged symptoms. Nevertheless, since commencing SIT his need for rescue medication (oral antihistamines and inhaled β_2 -agonists) had vanished. This way of reporting an effect of SIT has previously been shown to be statistically significant [22]. None of the patients reported essential allergic symptoms to grass pollen.

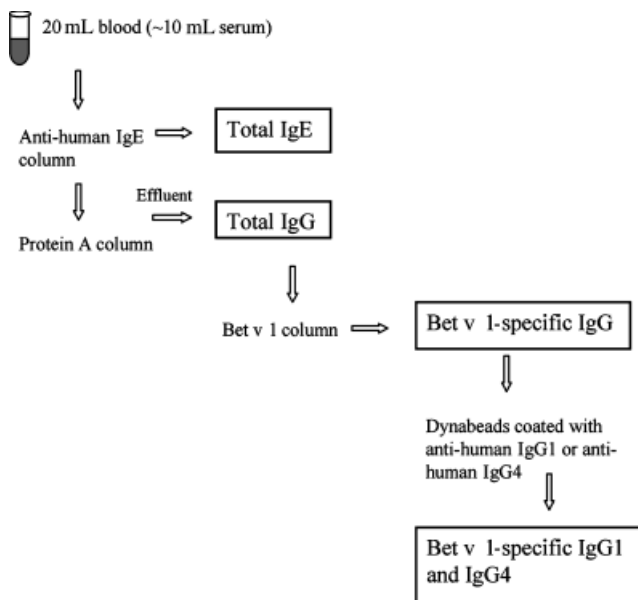


Fig. 1. Procedure for purification of antibodies from serum samples by affinity chromatography. The first 10 mL serum is run on an anti-human-IgE column to purify total IgE. The effluent from this column is run on a protein A column to purify total IgG. The fractions containing IgG are run on a Bet v 1 column to achieve Bet v 1-specific IgG. To the positive fractions magnetic beads coated with either anti-human IgG1 or anti-human IgG4 are added to purify Bet v 1-specific IgG1 and IgG4.

The study was approved by the local ethics committee of Copenhagen, Denmark, and written informed consent was obtained from all patients prior to admission to the study.

Purification of total immunoglobulins E and G from serum

Total IgE and IgG were purified by affinity chromatography from the 20 individual sera from the enrolled patients (Fig. 1).

Five milligrams of anti-human IgE (ATCC clone HB 121) was coupled to a sepharose 4B column (Amersham Biosciences, Bucks, UK) by following the instructions of the manufacturer. Ten millilitres serum with NaCl at a final concentration of 0.5 M was applied to the affinity column equilibrated with 100 mM phosphate buffer, 0.5 M NaCl, pH 8.0. Bound human IgE was eluted with 0.1 M citric acid. Fractions containing IgE were pooled, buffer was changed to 100 mM phosphate, pH 8.0, and the antibodies were concentrated to ~2 mL using Ultracel Amicon YM30 Ultrafiltration Discs (Millipore, Billerica, MA, USA).

The effluent from the anti-IgE column was loaded on a protein A column (Amersham Biosciences) for purification of total IgG. Fractions containing IgG were pooled, buffer was changed to 100 mM phosphate, pH 8.0, and the antibodies were concentrated to ~25 mL using Ultracel Amicon YM30 Ultrafiltration Discs (Millipore).

Purification of Bet v 1-specific immunoglobulin G from total Immunoglobulin G

One milligram of Bet v 1 (Biomay, Vienna, Austria) was coupled to sepharose 4B (Amersham Biosciences), and protein A-purified total IgG was applied on the column as described above for total IgE purification. Fractions containing Bet v 1-specific IgG were pooled and neutralized by addition of 0.1 M borate buffer, pH 10.0, and the antibodies were concentrated to ~3 mL using Ultracel Amicon YM30 Ultrafiltration Discs (Millipore).

Purification of Bet v 1-specific immunoglobulin G1 and Immunoglobulin G4

For purification of Bet v 1-specific IgG1 and IgG4, M-280 Tosylactivated Dynabeads (Dynal, Oslo, Norway) were coated with mouse anti-human IgG1 and IgG4, respectively (Biogenesis, Poole, UK).

Dynabeads coated with anti-human IgG4 were added to Bet v 1-specific IgG and incubated for 30 min at room temperature with slow tilt rotation and washed five times with PBS. The Bet v 1-specific IgG4 were eluted with 400 μ L 100 mM citric acid, and neutralized with 1.6 mL 0.1 M borate, pH 10.0. Dynabeads were washed four times in PBS and reused.

Subsequently, Dynabeads coated with anti-human IgG1 were used for isolation of Bet v 1-specific IgG1 from IgG4-depleted Bet v 1-specific IgG. The eluted antibodies were concentrated to ~500 μ L and the buffer was changed to PBS, pH 7.4, using a Centricon YM-10 Centrifugal Filter Unit (Millipore).

Determination of the concentrations of the purified antibodies

To measure the concentration of the purified antibodies, different ELISA assays were used. IgE was measured by coating an ELISA maxisorp plate (Nunc, Roskilde, Denmark) with mouse anti-human IgE (Zymed, San Francisco, CA, USA) diluted 1:4000 16 h at 4 °C. After blocking with 2% skim milk in PBS, IgE purified from the 20 serum samples diluted 1:10 was added. To allow quantification, a known standard human IgE (Biogenesis) was added in separate wells varying the concentrations. The bound IgE was detected with HRP-conjugated goat anti-human IgE (Serotec, Oxford, UK) diluted 1:3200. Between each 1 h incubation the plate was washed three times in PBS supplemented with 0.1% (v/v) Tween 20. Following the last incubation, staining was performed using TMB+substrate (Kem-En-Tec, Copenhagen, Denmark) stopped by adding 1 × volume of 0.2 M H₂SO₄; the optical density was measured at 450 nm.

IgG1 and IgG4 were measured by coating an ELISA maxisorp plate (Nunc) overnight at 4 °C with purified Bet v 1-specific IgG1 or IgG4 diluted 1:10 and a known standard of human IgG1 (Serotec) and IgG4 (Nordic Immunologicals, Tilburg, the Netherlands), respectively. After blocking with 2% (w/v) skim milk in PBS, the bound IgG1 or IgG4 was detected with mouse anti-human IgG1 (Biogenesis) or mouse anti-human IgG4 (Biogenesis) diluted 1:1000, biotin-conjugated rat anti-mouse IgG1 diluted 1:2000 (DakoCytomation, Glostrup, Denmark), and HRP-conjugated streptavidin (KPL, Gaithersburg, MD, USA) diluted 1:1000. Washing and staining were performed as described above.

Surface plasmon resonance (Biacore)

Recombinant Bet v 1 (Biomay) was immobilized on a CM5 biosensor chip using the *N*-hydroxy-succinimide (NHS)/*N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) kit (Biacore, Uppsala, Sweden) yielding a surface of ~1000 RU. The protein was immobilized at a concentration of 15 µg/mL in 10 mM sodium acetate, pH 4.0. Purified antibodies were injected non-diluted or 2-fold diluted in HBS-EP running buffer (10 mM HEPES, 3.4 mM EDTA, 150 mM NaCl), 0.05% (v/v) surfactant P20, pH 7.4). Serum samples were injected after a 20-fold dilution in HBS-EP running buffer and antibodies binding to Bet v 1 were analysed using a flow rate of 4 µL/min. The kinetics of binding was analysed using the BIAevaluation software.

Statistics

All data were analysed using the statistical software program GraphPad InStat version 3.0. The baseline characteristics of

the SIT and the non-SIT group were compared using Fisher's exact test for categorical variables and by the Mann–Whitney *U*-test for continuous variables. The Mann–Whitney *U*-test was also used for comparison between the two groups of all the obtained data. The Spearman test for correlations was used for comparison of VAS and the binding affinities.

Results

Clinical and paraclinical tests

Table 1 shows the result of different clinical and paraclinical tests on serum between the SIT-treated and the non-SIT-treated group of patients. There is no statistical difference in the amount of total IgE and Bet v 1-specific IgE in the serum in the two groups and the same is the case for the weal diameter in a SPT to *B. verrucosa*. Symptom severity was reported as significantly lower in the SIT group than among untreated patients.

Purification of human antibodies

The amount of total IgE purified from 10 mL serum from each patient varied from 40 ng to 14.5 µg as measured by ELISA. The Bet v 1-specific IgG1 amount measured by ELISA varied from 3 to 350 ng, and Bet v 1-specific IgG4 showed a variation from 10 to 600 ng of purified IgG4 from each patient serum sample. Yields varied from 2% to 50% of the original antibody amount.

Analysis of the antibody–Bet v 1 interaction

The apparent binding affinities (K_a) to Bet v 1 were measured by surface plasmon resonance. The concentration of specific IgE used for calculation of the K_a was based on the percentage of specific IgE in relation to total IgE measured in the CAP assay (Table 1) giving rise to binding affinities of Bet v 1-specific IgE at ~10⁹–10¹¹ M⁻¹ (Table 2). Bet v 1-specific IgG1 have affinities in the range ~10⁹–10¹¹ M⁻¹, and Bet v 1-specific IgG4 have affinities in the range ~10⁸–10¹¹ M⁻¹. For 10 out of 20 patients, the K_a for IgG1 has the highest value, and for four out of 20 patients, IgG4 has the highest affinity. There was no correlation between the binding affinities of IgE, IgG1 or IgG4.

Table 2 shows the median of the different on and off rates plus the binding affinities. The on rates (k_{on}) were determined from the association phase of the binding curve, whereas the off rates (k_{off}) were determined from the dissociation phase. The larger the on rate, the faster is the binding and in combination with a low off rate, antibodies of high affinity/avidity are achieved. It can be seen that there are no statistically significant differences between the SIT and the non-SIT group for either total IgE, Bet v 1-specific IgG1 or Bet v 1-specific IgG4. However, the SIT group has a significantly higher R_{max} value than the non-SIT group.

Affinity/avidity in relation to symptoms

In Fig. 2, the affinities to Bet v 1 of Bet v 1-specific IgE, IgG1 and -IgG4 for each patient are plotted against the symptoms of the patients.

Table 1. Results of clinical and paraclinical tests on serum

	SIT, <i>N</i> = 10	Non-SIT, <i>N</i> = 10	<i>P</i> -value*
Total IgE median (kU/L)	136 (34–398)	92 (40–365)	>0.06
Specific IgE median (kU/L)	41.1 (17.7–76.4)	22.6 (2.45–77.4)	>0.1
SPT diameter median	7.0 (4–14)	6.75 (4–9.5)	>0.2
VAS2002 median	26 (1–75)	59.5 (25–82)	0.04

*Mann–Whitney test.

SIT, specific immunotherapy; SPT, skin prick test.

Table 2. Surface plasmon resonance results for the SIT group and the non-SIT group

	SIT, <i>N</i> = 10	Non-SIT, <i>N</i> = 10	<i>P</i> -value*
$R_{\max}$ (serum) median/RU (range)	1940 (679–4680)	134.05 (0–493)	<0.0001
$k_{\text{on}}(\text{IgE})$ median/s (range)	4.07×10^5 (7.32×10^4 – 2.38×10^7)	5.91×10^5 (5.52×10^4 – 2.46×10^7)	>0.7
$k_{\text{off}}(\text{IgE})$ median/M s (range)	9.60×10^{-5} (9.18×10^{-6} – 3.21×10^{-4})	1.82×10^{-4} (6.94×10^{-6} – 1.75×10^{-3})	>0.4
$K_a(\text{IgE})$ median/M (range)	1.03×10^{10} (9.58×10^8 – 1.07×10^{11})	5.17×10^9 (1.01×10^9 – 1.82×10^{11})	>0.6
$k_{\text{on}}(\text{IgG1})$ median/s (range)	2.36×10^7 (1.55×10^6 – 1.07×10^8)	3.35×10^7 (1.74×10^6 – 1.13×10^8)	>0.6
$k_{\text{off}}(\text{IgG1})$ median/M s (range)	5.19×10^{-4} (1.98×10^{-4} – 4.04×10^{-3})	4.49×10^{-4} (8.61×10^{-5} – 9.66×10^{-4})	>0.6
$K_a(\text{IgG1})$ median/M (range)	5.04×10^{10} (7.00×10^8 – 5.40×10^{11})	4.86×10^{10} (1.37×10^{10} – 3.15×10^{11})	>0.4
$k_{\text{on}}(\text{IgG4})$ median/s (range)	1.00×10^7 (1.02×10^5 – 2.97×10^7)	1.97×10^6 (4.26×10^4 – 4.15×10^7)	>0.3
$k_{\text{off}}(\text{IgG4})$ median/M s (range)	1.11×10^{-3} (7.21×10^{-5} – 1.85×10^{-3})	5.63×10^{-4} (9.30×10^{-5} – 1.04×10^{-3})	>0.1
$K_a(\text{IgG4})$ median/M (range)	7.08×10^9 (7.72×10^8 – 1.03×10^{11})	3.95×10^9 (1.91×10^8 – 7.51×10^{10})	>0.3

Apparent binding affinity (avidity) $K_a/M = k_a/k_d$ for the antibodies purified from persons allergic to birch pollen. The value is an average of the affinities of the polyclonal antibodies in the samples.

*Mann–Whitney test.

SIT, specific immunotherapy.

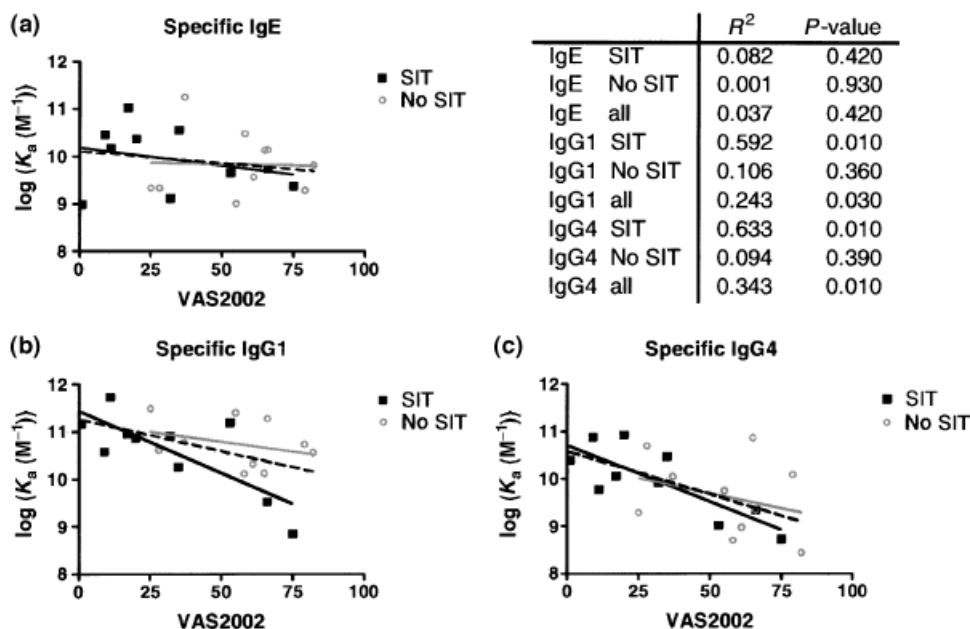


Fig. 2. The logarithm to the binding affinities, K_a , to Bet v 1 is plotted against the VAS2002 score of the individual patients. The linear correlation is shown; specific immunotherapy (SIT), black; no SIT, grey; all, dashed. The Spearman correlation test was applied. (a) Bet v 1-specific IgE, (b) Bet v 1-specific IgG1, (c) Bet v 1-specific IgG4.

Between the affinities of the specific IgE and the VAS2002 score, there is no correlation in any of the groups. For Bet v 1-specific IgG1, there is a correlation showing that the higher antibody affinity the lesser symptoms. The correlation is not statistically significant for the non-SIT group, but when looking at all patients combined, the correlation remains significant. Bet v 1-specific IgG4 shows the same pattern. In the non-SIT group, there is no statistical correlation but the Spearman correlation test shows that in the SIT group there is a correlation, showing that the patients with the lowest

symptom scores have the antibodies with the highest affinities, a trend that is true when looking at all patients. Between the amounts of Bet v 1-specific IgG1 or IgG4 and the VAS2002 score, there is no correlation (data not shown).

Discussion

The only clinical and paraclinical differences we observe between the SIT group and the non-SIT group is a

significantly lower VAS symptom score. These observations are in agreement with previous results reported from studies investigating patients before and after SIT treatment. Post-season VAS scoring has been shown to be reduced in groups having received SIT [23]. Furthermore, an increased level of allergen-reacting IgG4 has been found by several investigators and the majority of 'blocking antibodies' induced by SIT has been shown to belong to this subclass [9, 24, 25]. We do not see a lower total IgE and allergen-specific IgE level in the SIT-group. On the contrary, the levels are insignificantly higher in the SIT group. The response to SIT is characterized by an initial increase followed by a slow decline in total and specific IgE [26–28] but there is no definite agreement about the amount of IgE after SIT.

More than 95% of the patients suffering from IgE-antibody-mediated hypersensitivity to birch pollen produce IgE directed against the major birch pollen allergen Bet v 1, and about 60% of these react exclusively with this allergen [29, 30]. We use the Bet v 1a isoform in our binding studies. There are at least 20 isoforms of Bet v 1 in birch pollen extracts, and proteins homologous to Bet v 1 are present in pollen from other trees of the Fagales order, and in fruits and vegetables [31, 32]. However, it has been shown that although the different isoforms express exclusively as well as shared epitopes [33], one isoform may substitute natural Bet v 1 in degree of binding to IgE [21, 30, 33, 34]. This may be because of the statement that a single isoform (Bet v 1a) is dominant in Bet v extracts [35, 36]. In our studies the polyclonal antibodies have been purified; consequently, such differences will most likely not be observable although we use one recombinant isoform (Bet v 1a) in our studies instead of the natural Bet v 1.

As the amount of total IgE and allergen-specific IgG in human serum is extremely low, it has previously been necessary to use huge amounts of serum to purify enough antibodies to use for analysis of allergen-antibody binding. Total human IgE and Bet v 1-specific IgG1 and IgG4 were purified from only 10 mL serum from each individual patient giving enough material for further analysis. Although the achieved amounts of antibodies purified from each patient are low, one of the advantages of the surface plasmon resonance technique is that very small amounts of protein are enough for making kinetic binding measurements [37]. Furthermore, no labelling or reporter groups that might influence antibody-antigen interaction are required. When citric acid was used for regeneration of the Biacore chip after serum injection, it removed all antibodies binding to Bet v 1, as the refractive index on the surface of the chip was unchanged from before the injection (data not shown). As citric acid was also used for elution of Bet v 1-specific IgG from the Bet v 1 column, all antibodies were probably eluted, and the antibodies with the highest affinity were not retained on the column.

Our surface plasmon resonance data show that birch pollen-allergic patients receiving allergen-specific immunotherapy have more antibodies in their serum binding to Bet v 1 (high $R_{\max}$). As the amount of specific IgE is not statistically different from the non-treated group, these antibodies probably belong to the IgG subclass. This is totally in agreement with previous observations of the induction of blocking IgG antibodies of the IgG4 subclass after immunotherapy [6, 7].

We do not see any significant differences in binding affinities to Bet v 1 of purified human IgE, allergen-specific IgG1 or IgG4, between patients treated with SIT or not. It has previously been shown that the induction of affinity-matured IgG does not account for the reduced binding of IgE to Bet v 1 but that the binding reduction is mainly explained by increased amounts of IgG [21], i.e. a qualitatively similar ability of the anti-Bet v1 IgG antibodies of individuals to block Bet v 1 binding to IgE. Previously, it has been shown that there are differences in the specificity of antibodies from healthy and allergic individuals and the epitopes that are recognized by these antibodies [17–19].

We have shown that although there are no affinity differences between anti-Bet v 1 antibodies from SIT-treated vs. untreated patients, a correlation between the affinity of IgG1 and IgG4 and degree of symptoms exists; thus the higher the affinity for IgG1 and IgG4, the less the symptoms for the individual patient as estimated by the VAS score. This is in agreement with the above observation [18] that different specificity leads to different symptoms. Our results may indicate that it is not only the specificity of the antibodies that has an influence on the symptoms but also the affinity. The correlation between affinity and VAS score is significant for the allergen-specific IgG1 and IgG4 from the SIT-treated group but the same tendency is seen for the non-SIT IgG4 and also for allergen-specific IgG1 from both groups although it is not significant. A longitudinal study with the same patients before and after SIT, or a larger patient group could have increased the significance, since when looking at all 20 patients the correlation becomes significant for both IgG1 and IgG4. However, the difference in IgG affinity cannot explain all the experienced symptoms, as the R^2 values are relatively low in spite of a highly significant correlation.

In particular, for patient number 8 the immunotherapy with birch pollen extracts had no effect. This is also the person from the SIT group showing the highest VAS score and the lowest Bet v 1 affinities of the allergen-specific IgG1 and IgG4. This fits with the above theory that there is a correlation between a few allergy symptoms and a high allergen affinity.

In conclusion, we have shown that the amount of blocking antibodies is higher in a group of patients treated with SIT compared with a non-SIT-treated group, that the affinity of Bet v 1-specific IgE, IgG1, and IgG4 is probably not influenced by immunotherapy, but that those patients having antibodies with blocking capability with a high affinity also show less symptoms independent of immunotherapy or not.

Acknowledgements

C. G. J. was supported by the Danish Academy of Technical Sciences, and U. B. by the Danish Allergy Research Centre (DARC).

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