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A double-blind placebo-controlled birch allergy vaccination study II: correlation between inhibition of IgE binding, histamine release and facilitated allergen presentation

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Clinical and Experimental Allergy

Summary

Background The pathogenesis of IgE-mediated allergic disease is closely related to the production of T-helper type 2 (Th2) cytokines, which lead to IgE production pivotal for activation of mast cells and basophils. Proliferating T cells along with eosinophils expanded and attracted by Th2 cytokines are major contributors to the late-phase reaction. The activation of these Th2 cells is strongly enhanced by CD23-mediated IgE facilitated allergen presentation (FAP).

Objective The present study aims to investigate the effect of specific immunotherapy (SIT)-induced allergen-specific non-IgE antibodies (blocking antibodies) on IgE binding to allergen, histamine release (HR) and CD23-mediated allergen uptake in antigen-presenting cells.

Methods Competition between IgE and non-IgE for allergen binding was studied by Advia Centaur antibody measurements, passively sensitized basophils were used to study HR and IgE-facilitated binding of allergen to B cells (FAP) was studied by flow cytometry. FAP measurements were performed both with and without the addition of a reference IgE serum, which was included to obtain optimal complex formation. The serum samples were obtained from birch pollen immunotherapy ($n = 21$) or placebo control patients ($n = 21$) before and after 1 and 2 years of treatment.

Results Statistically significant reduction of all parameters investigated was observed after 1 year of treatment and the effect was maintained during the second year of treatment. There was a clear correlation between the two FAP measurements and between each of them and the level of T cell activation reported upon previously. Moreover, strong correlations were found between changes in FAP, IgE binding and HR.

Conclusion The present study clearly demonstrates that SIT induces changes in the composition of serum antibodies that inhibit IgE binding, HR and FAP to a similar extent. This suggests that these measurements, individually or in combination, may be used to monitor the immunological effect of SIT, even though direct correlations to changes in clinical parameters could not be demonstrated.

Keywords allergy vaccination, Bet v 1, blocking antibodies, facilitated antigen presentation, histamine release

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Introduction

Specific immunotherapy (SIT) with allergen extracts has been practiced for almost a century and is currently the only routinely administered antigen-specific immunomodulatory treatment given for any immunological disease

[1]. SIT is a highly effective treatment of IgE-mediated disease and clear effects are noted with respect to symptom/medication scores, early- and late-phase skin responses and increased allergen threshold levels in provocation tests [2, 3]. Immunotherapy has been shown to confer long-term efficacy several years after withdrawal

[4], and in children, immunotherapy has been shown to prevent onset of new sensitizations and to reduce progression of rhinitis to asthma [5, 6].

Even though the precise immunological mechanisms underlying SIT are still a matter of debate, several immunological effects have been described. In general, specific serum IgE levels do not decrease after SIT but rather show a transient increase, and a significant increase in allergen-specific IgG antibodies has been reported from various investigations [7–10]. Changes in cellular reactivity as a result of SIT include a decreased influx of activated eosinophils and activated CD4⁺ T cells into target tissues after allergen exposure [11] and a decreased number of circulating basophils [12]. A shift in the production of cytokines from T-helper type 2 (Th2) to Th0/Th1 type [13, 14], a decreased proliferative response [14] and an increased production of the regulatory cytokines IL-10 [15] and/or TGF- β [16, 17] have been demonstrated for allergen-specific T cells in different allergen systems. In line with this, increased production of IL-12 and IL-10 by antigen-presenting cells (APCs) has also been reported [18, 19].

It has been demonstrated by several investigators that SIT-induced IgG in individual patients can inhibit histamine release (HR) from basophils *in vitro* [20–23]. Similarly, blocking of HR has been shown with monoclonal human Bet v 1-specific IgG1 [24]. The protective effect of these 'blocking antibodies' was brought forward more than 70 years ago by Cooke and colleagues, who demonstrated that serum from patients treated with SIT inhibited the early-phase reaction in the skin (reviewed by Flicker and Valenta [25]).

These immunological changes seen as a direct result of SIT clearly suggest that the clinical effect of the treatment depends on its allergen-specific modulation of the immune system. The individual immunological changes that SIT induces, however, cannot completely explain the clinical changes described after SIT. This suggests that if key immunological changes directly related to the clinical improvement are sought, such changes should be involved in mechanisms connecting different components of the immune response, i.e. antibodies, APC, T cells and effector cells. Such an interplay has been demonstrated in IgE-facilitated allergen presentation (FAP) where CD23-mediated uptake of allergen IgE complexes in the APC enables T cells to be activated at extremely low allergen concentrations [26, 27]. Similarly, changes in the level of mast cell and basophil activation may also influence T cell activation and Th1/Th2 differentiation through changes in the mediator release or the cytokine environment at the site of allergen exposure [28]. Moreover, when the 'adjuvant' effect of IgE and IgG is investigated in mouse models, it is clear that the overall nature of the response is highly dependent on the signals induced by feedback mechanisms via Ig receptors (reviewed by Hjelm *et al.*

[29]). Thus, changes resulting in a reduction of IgE-allergen interaction may be key events that may affect various parts of the allergic reaction and thereby eventually lead to the clinical effect of the treatment.

Changes in allergen-specific non-IgE antibody titres after SIT show only poor correlations to clinical data such as symptom and medication score [30]. This may in part be explained by the lack of baseline values for clinical readouts in most trials resulting in the lack of delta-values, IgG assay systems addressing both blocking and non-blocking antibodies as well as the use of less well-defined allergen preparations for the analyses, measuring antibodies specific to non-allergens. Moreover, this may also reflect that not all non-IgE antibodies are relevant for the allergic response depending on specificity and affinity. Interestingly, some antibodies might even increase symptoms as demonstrated for SIT-induced Bet v 1-specific IgG [24, 31]. However, the enhanced binding to membrane-bound allergen induced by allergen-specific IgG observed in these studies is puzzling and needs to be accounted for in subsequent studies.

Taken together, the nature of the SIT-induced antibody response makes it highly important to investigate the effect of the relevant blocking antibodies in assays based on the competition between IgE and non-IgE antibodies. Furthermore, the competition between these antibodies may have a different impact on the binding of individual IgE molecules to the allergen, establishment of allergen IgE complexes, followed by binding to the low-affinity receptor (CD23) and on IgE-allergen interaction when the IgE is bound to the surface of an effector cell.

In the present study, the effect of SIT-induced changes in serum components (i.e. blocking antibodies) on IgE binding to allergen, HR from passively sensitized basophils, and binding of allergen-IgE complexes to CD23⁺ B cells was investigated to determine the influence of SIT on different aspects of IgE interactions in the allergic response (for simplicity, the abbreviation FAP will be used for both allergen binding to B cells and T cell activation). In addition, the relation between these measurements was investigated to evaluate whether the effect of SIT is similarly reflected in different IgE-dependent reactions.

Materials and methods

Patients and treatment

The study was designed as a randomized double-blind, placebo-controlled study of specific allergy vaccination in patients allergic to birch pollen and performed in Gothenburg, Sweden, from autumn 1996 to summer 1998. The clinical results of the study are reported in detail elsewhere [32]. In brief, 49 patients with a history of rhinoconjunctivitis with or without mild to moderate asthma to birch pollen were recruited for the study. The patients

were randomized to treatment with either birch pollen extract (*Betula verrucosa*, Alutard SQ[®], ALK-Abelló A/S, Denmark) or placebo. The maintenance dose (100 000 SQ-U) was reached in 7 weeks using a clustered updosing schedule of weekly injections of three or two doses, each separated by 30 min. The patients were treated for at least 18 months in the period from December or January 1996/1997 to July 1998 covering the birch pollen seasons of 1997 and 1998.

Allergens and control sera

Sera from the actively and placebo-treated birch-allergic patients undergoing SIT were obtained from before start of treatment, after 8–10 months of treatment and by the end of the study after 18–20 months of treatment (1996, 1997 and 1998). All sera were blinded, and clinical data were not available to the technicians at the time of the individual analyses. Sera from 21 actively treated and 21 placebo-treated patients were available in sufficient amounts for the study. Bet v 1 was purified as described previously [33].

Immunoglobulin E inhibition

The principle of the assay for non-IgE antibodies blocking IgE binding: All samples were tested on an Advia Centaur instrument [34] using two different assay procedures: one 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), and one excluding 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.

Briefly, the two measurements are performed as follows:

Wash assay. Serum sample is dispensed and solid phase anti-IgE is added [Para Magnetic Particles (PMP) covalently coupled with murine anti-human IgE antibodies]. Subsequently, the PMPs are washed and resuspended in wash buffer (Bayer Diagnostics, Newbury, UK), followed by stepwise addition of biotinylated allergen and acridinium ester-labelled Streptavidin. Finally, the PMPs are separated from the supernatant on a magnet, the fluid phase is aspirated and the particles are resuspended in wash buffer. This final separation procedure is repeated three times and subsequently the readout reaction is primed by addition of hydrogen peroxide in nitric acid. The light pulse recorded by the Advia Centaur instrument (relative light units, RLU) is generated after the addition of sodium hydroxide. The RLU responses are converted into dose (kU/L) via an instrument standard curve.

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 RLU and can be converted to a dose (kU/L) by the instrument standard curve. The ratio between the readout from the two assay procedures is a measure of the interference/inhibition by non-IgE antibodies on the binding of IgE to the allergens.

Histamine release

HR assays were performed with basophils in freshly isolated peripheral blood mononuclear cells (PBMC) from non-atopic donors. The cells were stripped for IgE by treatment with lactic acid and subsequently sensitized with pre-IT serum from the individual patients as described by Kleine et al. [35]. The allergen preparations were diluted in Pipes buffer pH 7.4 containing 0.1% w/v HSA, mixed with pre-heated pre- or post-IT sera and incubated with pre-heated sensitized basophils (PBMC) at 37 °C for 30 min. The incubation was performed with PBMC concentration corresponding to whole blood diluted 1 : 10, serum concentration 2.5% w/w and allergen concentrations ranging from 250 to 0.025 ng/mL.

After a 30-min incubation at 37 °C, followed by centrifugation, the supernatants were isolated and stored at – 20 °C until analysed for released histamine with Histamine ELISA (Enzyme Immunoassay kit IM2015, Immuno-tech, Marseille, France) according to the recommendation of the manufacturer. The release of histamine was measured as absorbance at 405 nm and reported as nm estimated from a standard curve. A serum from an IT-treated patient (> 3 years, birch pollen Alutard SQ[®]) was used as positive inhibitory control in all HR assays. All samples from the same patient, before/after immunotherapy, were analysed in the same experiment.

Epstein-Barr virus -transformed B cells

The EBV-transformed B cell lines were cultured in RPMI, 2 mM L-glutamine, 100 IU penicillin/streptomycin and 10% v/v heat-inactivated fetal calf serum (Gibco, Paisly, UK) under sterile conditions at 37 °C in a humidified atmosphere of 5% v/v CO₂.

Facilitated allergen-immunoglobulin E binding to B cells (facilitated allergen presentation) assays

Both the FAP assay and the FAP inhibition assay used in the present investigation involve the formation of allergen-IgE complexes by incubation of different concentrations of birch pollen (*B. verrucosa*) allergen extract with individual IT sera or with a combination of an indicator serum containing high concentrations of birch pollen-specific IgE (obtained from a single birch-allergic donor)

and the individual IT serum samples. In both assay systems, these allergen IgE complexes are allowed to bind to the surface of an EBV-transformed, high-CD23-expressing human B cell line and the binding is detected by fluorescence-activated cell sorting (FACS) detection of IgE-positive B cells and the assay principle is described in detail elsewhere [36–38].

Facilitated allergen presentation assay

Serum samples from the IT- or placebo-treated patients were pre-incubated in buffer alone or together with Bet v 1 (0, 0.3, 1.0 and 3.0 µg/mL) at 37 °C for 1 h (vol-allergen:IT-serum = 1+2) before adding the EBV-transformed B cells at 10⁵ cells per sample (vol-allergen:IT-serum:B cells = 1+2+1) and further incubation for 1 h at 4 °C. Cells were washed and surface binding of allergen–IgE complexes was detected by flow cytometry using an FITC-labelled anti-IgE polyclonal Ab (F0352, Dako, Glostrup, Denmark). All samples from the same patient, before/after immunotherapy, were analysed in the same experiment.

Facilitated allergen presentation inhibition assay

A stock indicator serum from a birch pollen-allergic donor containing high concentrations of allergen-specific IgE (> 100 kU/mL, Advia Centaur, ALK, Hørsholm, Denmark) was pre-incubated with Bet v allergen extract (0, 0.1 and 0.3 mg/ml) at 37 °C for 1 h alone (vol-allergen:indicator:buffer = 1+1+1) or in the presence of patient sera (vol-allergen:indicator:IT serum = 1+1+1) from patients undergoing immunotherapy or placebo treatment. Next, EBV-transformed B cells were added at 10⁵ cells per (vol-allergen:indicator:IT-serum:B cells = 1+1+1+1) sample and the samples were further incubated for 1 h at 4 °C before washing and detection of surface binding of allergen–IgE complexes by flow cytometry using an FITC-labelled anti-IgE polyclonal Ab (F0352, Dako). All samples were analysed in duplicate. All samples from the same patient, before/after immunotherapy, were analysed in the same experiment.

The stock indicator serum resulting in strong complex formation was used as positive control in all FAP assays and as standard serum in all FAP inhibition assays where a rabbit anti-Bet v serum was used as a positive inhibition control (not shown).

Data processing and statistic evaluation

The FAP, FAP inhibition and HR were performed with several dilutions of Bet v 1 and all response curves were evaluated before unblinding of the data to select a common allergen concentration for assay readout in each assay. 1.0 and 0.1 µg/mL were used to determine changes

in FAP (Fig. 2a) and FAP inhibition (Fig. 3a), respectively, while 0.25 ng/mL was used in the HR assay (Fig. 4a). Additional information might have been obtained for the HR assay if a shift in fitted curves or the allergen concentration inducing a certain response had been selected as readout. However, we found that the response of a known allergen concentration gave the most consistent determination of the effect of the blocking antibodies in the FAP assays as well as the HR assay used in the present study. Moreover, the sensitivity of basophils seems to be directly reflected in the ascending part of the dose–response curve when the cells are activated in the presence of IL-3, which enhances the level of histamine released.

HR and FAP data were normalized relative to the values obtained from the pre-treatment serum sample set to 100% for graphic representation and statistical analysis. IgE inhibition was expressed as relative values with the average pre-treatment value in the range of 1 and these values were used directly for graphic representation and statistical analysis. The statistical evaluation of all data was performed using non-parametric statistics such as the Friedmans test for repeated measurements within groups and the Kruskal–Wallis test between groups. Both tests were performed using Dunn's multiple comparisons test to evaluate differences between each time-point or between groups. All datasets were correlated to each other and to data on IgE, IgG4, T cell activation and symptom and medication scores as well as LPR reported upon previously from the present trial. Evaluations of significant correlations were performed using Spearmann's rank correlation test.

Approvals

Approvals from the Swedish Medical Products Agency and the Ethics Committee and signed informed consent were obtained before the start of the trial. The trial was conducted in accordance with the Helsinki Declaration and with good clinical practice.

Results

Summary of clinical results

The clinical results from the study are described in detail elsewhere [32]. The results demonstrate that there was a significant reduction in medication (72.1%) and a tendency in reduction of symptoms (38.2%) in the active group as compared with the placebo group in the season 1997 and significant efficacy of a 40% ($P=0.005$) reduction in symptom score and an 81.4% ($P=0.004$) reduction in drug consumption in the 1998 season. There was a highly significant difference in the severity of disease evaluated by a visual analogue scale in both 1997 ($P=0.0005$) and 1998 ($P=0.041$).

Significant reductions in late cutaneous responses to birch and specific allergen bronchial provocation test also documented the clinical effect of the treatment, while there was no detectable difference in the specific conjunctival provocation test.

Inhibition of immunoglobulin E-binding

The interference or inhibitory capacity of treatment-induced non-IgE allergen-specific antibodies was estimated as the ratio between IgE measured in the presence and absence of inhibitory antibodies.

The results shown in Fig. 1 demonstrate that the inhibitory capacity of treatment-induced non-IgE antibodies is almost completely unchanged in all individuals in the placebo-treated group (< 10% change compared with pre-treatment values). As a result of this, the average IgE inhibition did not change from the year 1996 to 1997 or 1998 in this group of patients. In contrast, only three patients in the actively treated group showed < 10% reduction in IgE binding over the treatment period resulting in a highly significant inhibition in this group ($P < 0.001$) after the first treatment year. The inhibition was sustained during the second treatment year, resulting in comparable average values in 1997 and 1998 ($P < 0.05$) even though some variation was seen from patient to patient. The different developments in the IgE inhibition in the placebo and the actively treated group led to significant differences in this parameter when 1997 and 1998 values were compared between groups ($P < 0.0001$ for both time-points).

Histamine release

Functional basophils are easily obtained in peripheral blood mononuclear cells and HR from these cells may be

used to represent the mechanisms of IgE-dependent activation of effector cells in the allergic response. When basophils are sensitized with IgE *in vitro* and activated in the presence of pre- and post-SIT sera, it is possible to monitor the effects of changes in allergen-specific antibodies on the effector cell level. The results represented in Fig. 2b are obtained from HR at 0.25 ng/mL Bet v 1 as representative of HR curves from the individual patients as seen in the example in Fig. 2a. In the placebo group, the individual curves varied somewhat during the treatment period but only two patients showed a strongly decreased HR in 1997, and one of them even returned to baseline values in 1998 as seen from Fig. 2b. The remaining patients in general showed unchanged or slightly increased values, ranging from a 20% decrease to a 40% increase in 1998, resulting in almost identical average HR values at all time-points investigated. On the other hand, the pattern for the HR curves from the actively treated patients was easily distinguishable in most cases, showing clearly reduced HR in 1997 and/or 1998. Only four patients showed < 20% reduction in HR in 1998, resulting in a minor overlap between the changes in HR values in the placebo and actively treated group and a highly significant difference ($P < 0.0001$ between groups in 1997 as well as 1998).

Facilitated allergen binding to B cells (facilitated allergen presentation) assays

The involvement of IgE in the presentation of allergen to T cells (FAP) has been investigated previously in these serum samples using B cell-induced T cell activation as the main readout [33]. The preceding binding of the allergen-IgE complexes to the B cell is easily detectable in flow cytometry [FACS-FAP or facilitated allergen binding (FAB)] [36, 38] and this method is used in the present study to monitor the effect of SIT.

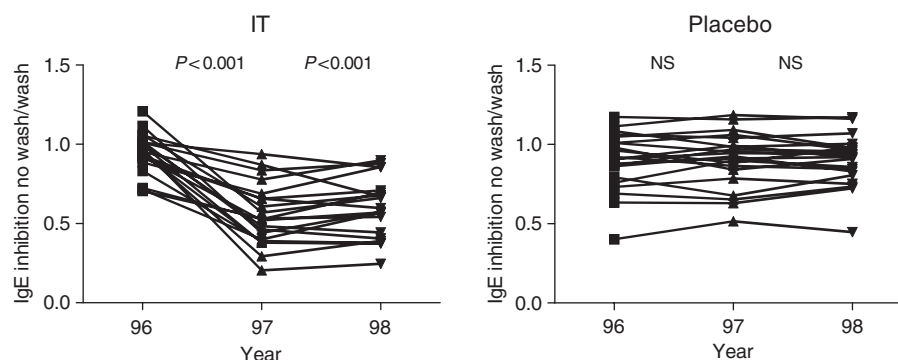


Fig. 1. Serum inhibition of IgE binding to allergen. Data are expressed as IgE inhibition (IgE binding to allergen in the presence of allergen-specific non-IgE antibodies/IgE binding alone) Year 1996 is before treatment, 1997 is after 8–10 months of treatment and 1998 is after 18–20 months of specific immunotherapy (SIT). Data from placebo-treated and actively treated patients are shown as indicated. The level of significance for the change from 1996 to 1997 and from 1996 to 1998 is indicated on the figure. NS, non-significant.

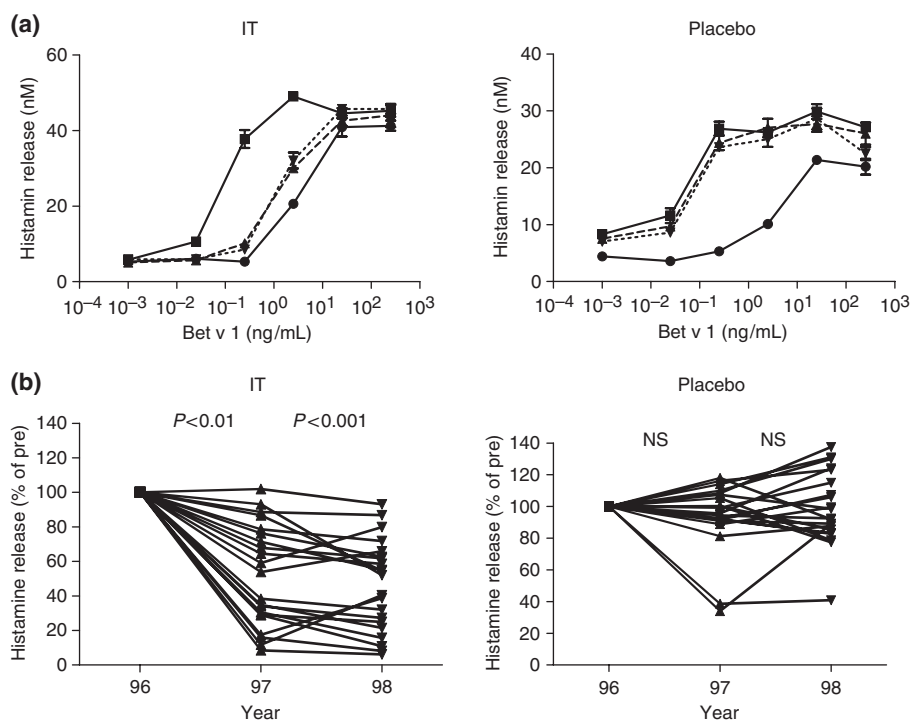


Fig. 2. Histamine release (HR). Treatment duration as described in Fig. 1 for placebo-treated and actively treated patients as indicated. A positive IT-control (IT-cont) patient was included in all HR assays IT-cont. (a) Dose-response curves from a placebo-treated and an actively treated patient. Squares with a solid line: 96 values, triangles with dashed lines: 97 values, inverted triangles with dotted lines: 98 values, filled circles with solid lines IT-control. Error bars indicate SEM values. (b) Data from all patients expressed as percent of the pre-treatment value. 0.25 ng Bet v 1 was used in the HR assay to calculate the changes in basophil activation. The level of significance for the change from 1996 to 1997 and from 1996 to 1998 is indicated on the figure. NS, non-significant.

Facilitated allergen presentation assay

Data were obtained from 31 patients; eight patients in the actively treated group and three patients in the placebo group did not show formation of detectable allergen-IgE complexes when this was attempted without the addition of indicator serum. Examples of the dose-response curves from individual patients are shown in Fig. 3a and data from one Bet v 1 concentration (1 µg/mL) from all patients can be found in Fig. 3b, which shows that very consistent reductions are found in almost all actively treated patients (range 7–78% in 1997 and 2–106% in 1998). The placebo-treated patients exhibit moderate variations, with FAP values around 100% in 1997 with a slight general increase in 1998 (range 16–175% in 1997 and 45–274% in 1998). The distribution of data leads to significant reductions in the actively treated group when comparing mean values from 1996 to 1997 and 1998 values, which is not found in the placebo group and a highly significant difference between groups in both 1997 ($P < 0.001$) and 1998 ($P < 0.0001$).

Facilitated allergen presentation inhibition assay

Data were obtained from all patients in the actively treated group as well as in the placebo group in FAP with

indicator serum. Examples of the dose-response curves from individual patients are shown in Fig. 4a and data expressed as percent of pre-IT from one Bet v 1 concentration from all patients can be found in Fig. 4b. Compared with the FAP described above, data were more consistent in both groups, leading to inhibition in all patients in the actively treated patients at both time-points (range 14–80% in 1997 and 13–83% in 1998). The placebo-treated patients exhibit some variation with FAP inhibition values in the range 63–144% in 1997 and 82–177% in 1998, and this distribution of data leads to significant reductions in the actively treated group over time, which is not found in the placebo group and a highly significant difference between the groups in both 1997 and 1998 for this analysis as well ($P < 0.0001$ for both time-points).

Correlations

Spearman's correlation coefficients were determined for the individual parameters and, as can be seen in Fig. 5a–c, highly significant correlations were found between the T cell activation measured previously [33] and both FAP measurements, between the two FAP measurements as well as between IgE inhibition, HR and FAP inhibition as shown in Fig. 6a–c. Significant correlations were not

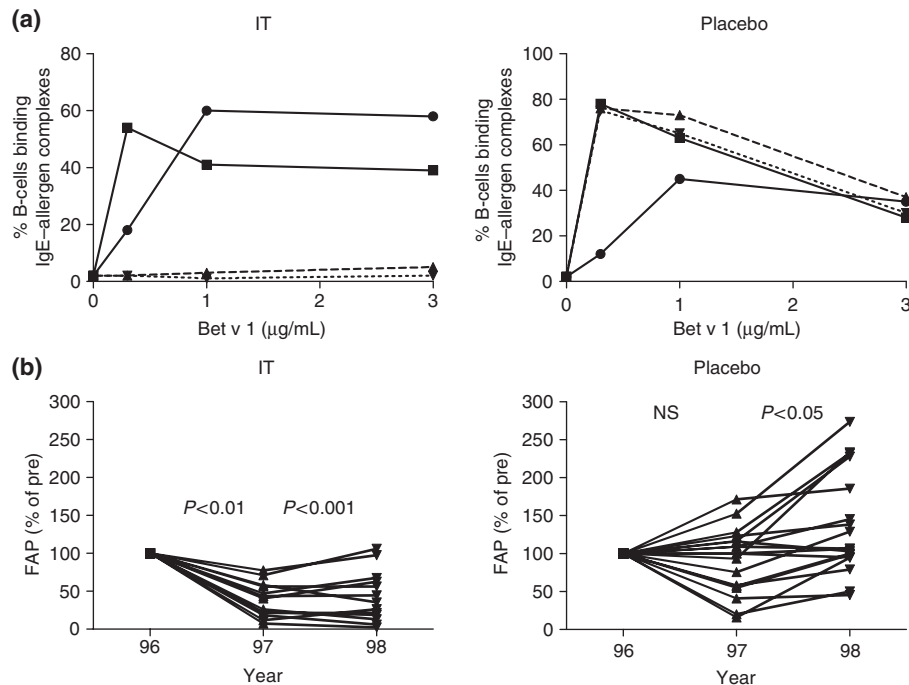


Fig. 3. Facilitated allergen presentation (FAP). Treatment duration as described in Fig. 1 for placebo-treated and actively treated patients as indicated. (a) Dose-response curves from a placebo-treated and an actively treated patient. A serum from a birch-allergic patient resulting in strong complex formation was used as a positive control in all FAP assays (cont). Squares with a solid line: 96 values, triangles with dashed lines: 97 values, inverted triangles with dotted lines: 98 values, filled circles with solid lines: FAP-control. (b) Data from all patients expressed as percent of the pre-treatment value. 0.3 μg/mL Bet v 1 was used to calculate changes in FAP. The level of significance for the change from 96 to 97 and 96 to 98 is indicated on the figure. NS, non-significant.

found for any of the parameters measured when related to symptom or medication scores, even though each parameter clearly differed between the treatment and placebo group as described above.

Discussion

The immunological changes associated with SIT have been described in various reports and the theories for the mechanisms behind the beneficial effects have developed extensively during the last decade [39]. Recently, the focus has been on T cells, especially the induction of regulatory T cells [40]. In this context, Robinson et al. [2] proposed a model for the effects of immunotherapy combining Th2 to Th1 deviation, Treg inhibition of allergen-specific T cell responses as well as the induction of allergen-specific non-IgE antibodies. But the specific effects of the non-IgE antibodies were not accounted for in the model and have only been sparsely investigated. We have previously described the effect of SIT-induced Bet v 1-specific IgG from individual patients on T cell activation via FAP in birch pollen allergy [27] and this has been confirmed with serum samples from larger studies for both birch [33] and grass [38, 41] SIT. The present study aims to investigate the effect of SIT-induced allergen-specific non-IgE antibodies (blocking antibodies) on IgE binding to allergen,

HR from passively sensitized basophils and binding of allergen-IgE complexes to CD23⁺ B cells (FAP assay). Moreover, the binding of allergen to B cells monitored by FACS was evaluated as a surrogate parameter for the actual allergen presentation leading to T cell activation.

The poor correlation between IgE titres and clinical manifestations [42, 43] as well as between the level of allergen-specific IgG antibodies and treatment effect [25] clearly suggests that such measurements are of little help in the prediction of symptoms or the evaluation of the effect of SIT in the individual patient. Competition between IgE and non-IgE antibodies for their binding to allergen is the simplest explanation for the immunological effect of SIT-induced non-IgE antibodies and may be used to link blocking antibodies to the clinical effect of the treatment. This competition can be evaluated directly in IgE inhibition studies [44, 45] and the effect of the competition on effector cell responses and T cell activation can be measured in basophil activation tests [21, 46] and FAP assays [33, 37], respectively. The changes induced in serum antibodies are clearly reflected in these measurements as can be seen from Figs 1–4 and the change is induced early in the course of treatment and sustained during the second treatment year. IgE inhibition and FAP inhibition seemed to show the most consistent changes while, for some patients, only slight inhibitions

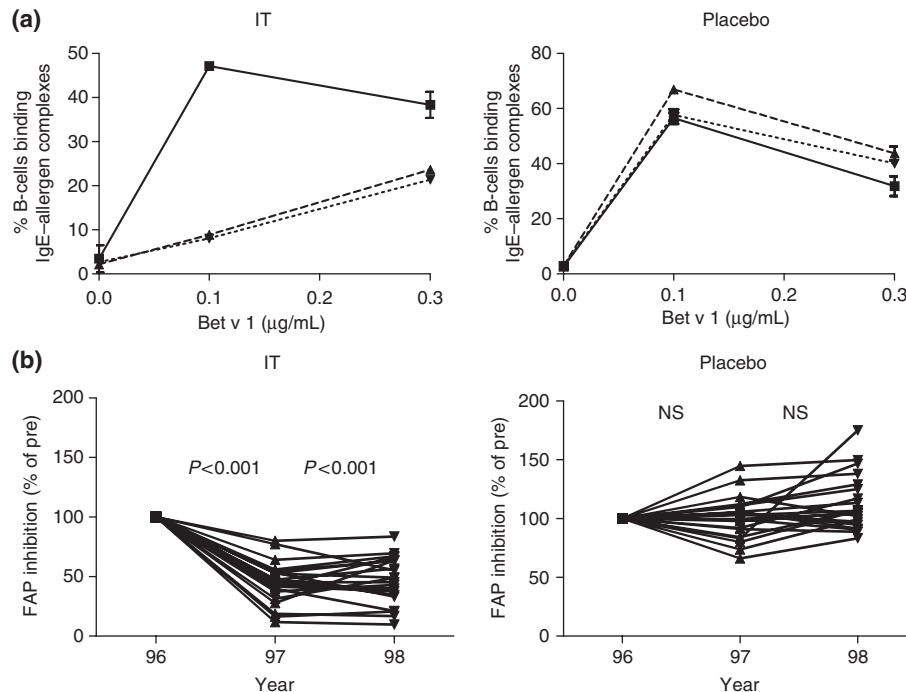


Fig. 4. Facilitated allergen presentation (FAP) inhibition. Treatment duration as described in Fig. 1 for placebo-treated and actively treated patients as indicated. A serum from a birch-allergic patient resulting in strong complex formation was used as indicator serum in all FAP inhibition assays. (a) Dose-response curves from a placebo-treated and an actively treated patient. Squares with a solid line: 96 values, triangles with dashed lines: 97 values, inverted triangles with dotted lines: 98 values. A rabbit anti-Bet v 1 IgG was included in all fluorescence-activated cell sorting-facilitated allergen presentation (FACS-FAP) inhibition assays as a positive control (not shown). (b) Data from all patients expressed as percent of the pre-treatment value. 0.1 µg/mL Bet v 1 was used to calculate changes in FAP inhibition. The level of significance for the change from 1996 to 1997 and from 1996 to 1998 is indicated on the figure. NS, non-significant.

were observed in HR and FAP. IgE inhibition shows completely unchanged values in the placebo-treated group, suggesting that the competition between IgE and non-IgE antibodies is unaltered during the treatment period in this group. On the other hand, fluctuations were observed in both HR and FAP inhibition assay and in the FAP assay in particular, which may reflect the variance of these cell-based assays. Previously published data [33] as well as the current IgE measurements (data not shown) do not show changes in the IgE levels, which could account for the increased FAP, FAP inhibition and HR values observed for some patients in the placebo group.

The observed immunological effects of immunotherapy have been indicated in various other studies where similar measurements have been performed individually and related to the changes in allergen-specific serum antibodies and/or clinical outcomes as reported upon previously also from this trial [33]. Basophil activation has until now primarily been performed in a few patients in different studies to stress the importance of the blocking antibodies on the effector cell activation [46–48]. Larger studies have demonstrated the effect of rush immunotherapy on basophil activation, but the effects of blocking antibodies have not been addressed directly in these studies [47, 49]. This effect has been confirmed in large patient groups in the

present study, underscoring that changes in serum antibodies may directly influence effector cell activation in the allergic response (Fig. 2).

To investigate the T cell antibody interaction further, IgE facilitated allergen binding to B cells (as a surrogate for FAP) was measured in two ways – with (FAP inhibition) or without (FAP) inclusion of an indicator serum, which is included to ensure that comparable complex formation is obtained in all the assays. It is evident that the inclusion of an indicator serum leads to more uniform data in the FAP inhibition studies (Fig. 4) and that it was not possible to induce detectable complex formation in 11 out of 42 patient sera without inclusion of the indicator serum (Fig. 3), suggesting that FAP inhibition should be preferred. The FAP measurements, primarily used in birch allergy [27, 33], may reflect the *in vivo* situation best with the relevant autologous serum IgE competing with SIT-induced non-IgE antibodies, while the FAP inhibition assay, preferred for grass allergy [37, 38, 41], may represent a more artificial situation that primarily reflects changes in allergen-specific non-IgE antibodies when they are competing with IgE from the indicator serum. However, the clear correlation between these two measurements seen in Fig. 5c implies that they can be used interchangeably, at least when well-defined single

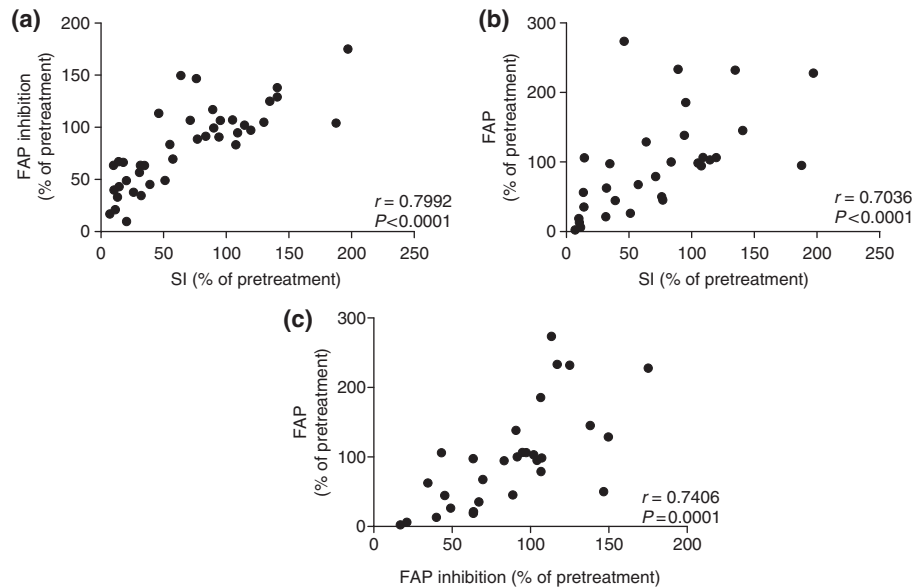


Fig. 5. Correlation between facilitated allergen presentation (FAP) and T cell activation (a), FAP inhibition and T cell activation (b) and FAP and FAP inhibition (c). Spearman's rank correlations coefficients and significance levels are shown in the figures. T cell activation data were obtained in a previous study [33] and are expressed as stimulation index (SI).

allergens are used in the assay procedures. Moreover, significant correlations were also demonstrated for each of these FAP measurements and the T cell activation reported upon previously [33], which demonstrates that the binding of allergen–IgE complexes to the B cell surface closely reflects the subsequent T cell activation, which directly represents FAP (Fig. 5a and b). Thus, the practical optimization of the assay procedure including an indicator serum leads to consistent data from all patient sera investigated and seems to reflect T cell activation well. Moreover, the FAP inhibition procedure was recently validated thoroughly for use in grass pollen allergy, which suggests that it may be generally applicable for evaluation of the immunological effect of allergen immunotherapy [37]. The abbreviation FAB was suggested for this specific assay procedure measuring facilitated allergen binding as a surrogate for FAP.

In general, when comparing IgE inhibition, HR and FAP assays, each assay has theoretical or technical advances such as a high degree of automation and straightforward interpretation of the IgE inhibition measurements, the reflection of effector cell responses directly involved in the immediate allergic response for the HR assay or the linking of T cell and antibody responses for the FAP assays. On the other hand, the clear correlations observed when these measurements are compared on the basis of data expressed as percent of pre-treatment values (Fig. 6) demonstrate that the changes induced in serum antibodies influence each of these parameters to the same extent, at least after more than 6 months of treatment. This suggests that one type of measurement is sufficient to monitor these immunological changes.

While these changes are clearly a result of SIT treatment, attempts made to correlate the immunological changes to clinical efficacy on the level of single patients generally led to poor correlations [25, 50, 51] and no significant correlations were found between each of the different measurements performed in the present study and symptom and medication scores in 1998 or LPR in 1997 (data not shown).

In general, the complexity of the mechanism behind allergic disease and the symptoms experienced by the patients may make it difficult to find valid biomarkers for the clinical effect of SIT. A more direct comparison might be based on the relationship between the various immunological changes and changes in symptom and medication scores as the clinical situation before start of treatment may influence both symptom score and drug consumption. It would be necessary to obtain baseline data on the clinical parameters to compare delta-values or the development in each parameter, but such data were not available in the current investigation. Alternatively, each immunological change may more closely reflect clinical changes observed at different time-points during up dosing or maintenance treatment as recently suggested by Wilcock and colleagues for HR, FAP, and allergen-specific IL-10 production when related to changes in skin prick test and late-phase reactions [52].

Conclusion

Immunological changes are frequently used as indicators of successful immunotherapy even though clear correlations between these measurements and clinical outcomes

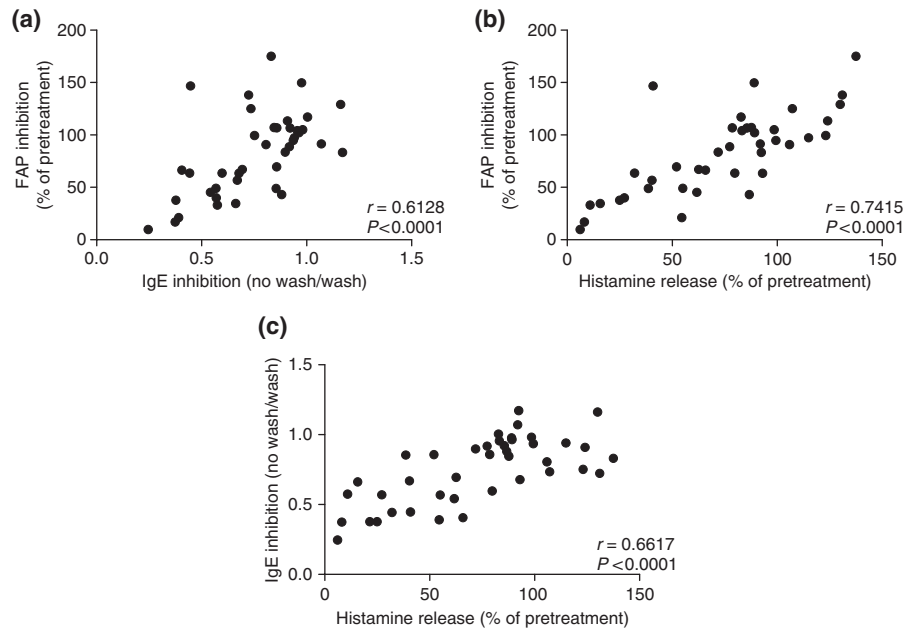


Fig. 6. Correlation between facilitated allergen presentation (FAP) inhibition and IgE inhibition (a), FAP inhibition and histamine release (b) and IgE inhibition and histamine release (c). Spearman's rank correlation coefficients and significance levels are shown in the figures.

have not been established to date. We confirmed that the inhibition of basophil activation, inhibition of IgE binding and inhibition of IgE-FAP are clear serum-induced effects of successful birch pollen immunotherapy when comparing actively treated and placebo patients. This strongly suggests that these measurements, one by one or in combination, may be used to monitor the effect of SIT.

Future studies investigating one or more of these immunological changes in large patient groups at different time-points during the course of immunotherapy may establish clear correlations with changes in symptom and medication scores.

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