

Functional rather than immunoreactive levels of IgG₄ correlate closely with clinical response to grass pollen immunotherapy

M. H. Shamji¹, C. Ljørring², J. N. Francis¹, M. A. Calderon¹, M. Larché³, I. Kimber⁴, A. J. Frew⁵, H. Ipsen², K. Lund², P. A. Würtzen² & S. R. Durham¹

¹Allergy and Clinical Immunology, National Heart and Lung Institute, Imperial College London, Part of the Medical Research Council and Asthma UK Centre for Allergic Mechanisms of Asthma, London, UK; ²ALK Abelló, Bøge Alle, Hørsholm, Denmark; ³ALK Abelló, Bøge Alle, Hørsholm, Denmark; ⁴Department of Medicine, Firestone Institute for Respiratory Health, McMaster University, Hamilton, ON, Canada; ⁵Faculty of Life Sciences, University of Manchester, Oxford Road, Manchester, UK; ⁵Department of Allergy & Respiratory Medicine, Royal Sussex County Hospital, Brighton, UK

To cite this article: Shamji MH, Ljørring C, Francis JN, Calderon MA, Larché M, Kimber I, Frew AJ, Ipsen H, Lund K, Würtzen PA, Durham SR. Functional rather than immunoreactive levels of IgG₄ correlate closely with clinical response to grass pollen immunotherapy. *Allergy* 2012; **67**: 217–226.

Keywords

biomarkers; blocking antibodies; facilitated allergen presentation; IgG₄; immunotherapy.

Correspondence

Prof. Stephen R. Durham, Allergy and Clinical Immunology Section, National Heart and Lung Institute, Faculty of Medicine, Imperial College London, Dovehouse Street, London, SW3 6LY, UK.
Tel.: +44 207 351 8024
Fax: +44 207 351 8949
E-mail: s.durham@imperial.ac.uk

Accepted for publication 4 October 2011

DOI:10.1111/j.1398-9995.2011.02745.x

Edited by: Thomas Bieber

Abstract

Background: Induction of allergen-specific IgG₄ antibodies is the most consistent immunological finding in immunotherapy trials. However, quantitative assessments of IgG₄ antibodies have not proven beneficial in evaluating clinical changes during or after immunotherapy. In the current study, we investigated the relationship between clinical outcome and allergen-specific IgG₄ titres or functional antibody responses following immunotherapy. We hypothesized that functional assays of serum IgG-associated inhibitory activity such as inhibition of IgE–allergen interactions (IgE-blocking factor) and inhibition of CD23-dependent IgE-facilitated allergen binding (IgE-FAB) correlate more closely with clinical outcome and may be biomarkers of clinical response.

Methods: In an 8-month dose–response randomized double-blind placebo-controlled study, 221 polysensitized subjects with severe seasonal rhinitis received Alutard SQ, *Phleum pratense* 100 000 SQ-U, 10 000 SQ-U or placebo injections. Serum specimens were collected before treatment, after up-dosing, during the peak season and at the end of the study. Allergen-specific IgG₄ titres and IgG-associated inhibitory activity were evaluated.

Results: A time- and dose-dependent increase in serum inhibitory activity for both the IgE-blocking factor and IgE-FAB was observed, which paralleled increases in grass pollen-specific IgG₄ antibodies. A modest but significant inverse relationship was demonstrated between postimmunotherapy serum inhibitory activity and combined symptom–rescue medication scores (IgE-FAB: $r = -0.25$, $P = 0.0002$; IgE-blocking factor: $r = -0.28$, $P < 0.0001$), whereas this was not observed for immunoreactive IgG₄ levels ($r = -0.11$, $P = 0.12$).

Conclusions: Functional assays of inhibitory IgG₄ and IgE-blocking factor may be more useful surrogates of clinical response than IgG₄. Whether these antibody effects may serve as predictive biomarkers of clinical efficacy in individual patients requires further investigation.

Abbreviations

FAB, facilitated allergen binding; FcεRI, high-affinity IgE receptor; FcεRII, CD23, low-affinity IgE receptor; IgE BF, IgE-blocking factor; IgG₄, immunoglobulin G₄; Non-IgE, antibody isotypes other than IgE; SIT, specific immunotherapy.

Grass pollen-specific immunotherapy (SIT) confers significant clinical benefits in patients with seasonal allergic rhinitis. These include symptom reduction, long-term remission and prevention of new sensitizations in children (1, 2). However, treatment is costly, lasts up to 3 years and may occasionally lead to systemic adverse reactions. Therefore, careful patient selection and early identification of responders are important to target intervention at those who will benefit and to exclude those who are less likely to respond to immunotherapy.

Surrogate endpoints could be of major importance for the development of new vaccines and for optimization of existing treatment regimes. In addition, clinical management of patients receiving therapy could be significantly enhanced if there were means to identify those who are most likely to respond, when to stop treatment and how to predict relapse. Hence, it is important to evaluate objective and clinically feasible surrogates and biomarkers, which could be used to design more efficient clinical studies and to guide and adjust therapy to improve symptom control for individual patients. Several small studies have demonstrated that allergen injection immunotherapy is associated with elevated concentrations of allergen-specific IgG antibodies, in particular IgG₄ antibodies, which have inhibitory activity (3–8). Functional IgG antibodies have potential to compete with allergen-specific IgE, inhibit basophil activation and prevent allergen presentation by B cells to T-cell clones (9–11). Additionally, the IgG₄ antibody subclass does not activate complement, does not form immune complexes and is nonanaphylactogenic as it is unable to cross-link Fcε receptors (12).

Changes in allergen-specific IgG antibody concentrations following injection immunotherapy have shown a poor correlation with clinical response as measured by symptom and medication scores (13, 14). These observations may be due in part to the lack of baseline values for clinical readouts in most clinical trials. Functional IgG assays involving competition between IgE and serum inhibitory antibodies such as blocking of IgE–allergen interaction (15) and inhibition of CD23-dependent IgE-facilitated allergen presentation (6, 16, 17) may possibly be more suitable surrogates to reflect efficacy. It is therefore important to investigate the effect of the relevant inhibitory antibodies in assays based on the competition between IgE and functional IgG antibodies (15, 16). The competition between these antibodies may have a different impact on the binding of individual IgE molecules to allergen, the degree of allergen–IgE complex formation and the ability of such complexes to bind to the low-affinity receptor (CD23) on antigen-presenting cells (APC). Similarly, these inhibitory antibodies may have different abilities to interfere with FcεRI-dependent events such as IgE–allergen interaction with surface receptors on mast cells, basophils and dendritic cells (18).

We have recently demonstrated that long-term clinical tolerance was associated with IgG₄-associated inhibitory activity against binding of IgE–allergen complexes to B cells, a surrogate of IgE-facilitated antigen presentation and activation of antigen-specific T cells (19). IgG₄-associated inhibitory activity rather than absolute concentrations of the IgG₄ antibody was associated with clinical tolerance, as reflected in maintained suppressed combined symptoms and the need for

rescue medication 2 years after discontinuation of injection immunotherapy. Depletion of IgG₄ resulted in reduction in serum inhibitory activity. Following these findings, we chose to assess the relationship between immunoreactive or functional allergen-specific IgG₄ and combined symptoms and rescue medication scores.

We hypothesized that high-dose subcutaneous grass pollen-SIT is associated with the induction of serum IgG-associated inhibitory activity. We further hypothesized that postimmunotherapy serum inhibitory activity, as determined by IgG₄ concentrations or functional assays, correlates with clinical response to immunotherapy, as reflected by reduced combined seasonal symptom and rescue medication scores (CSMS) and rhinoconjunctivitis quality-of-life scores. We examined serum inhibitory antibodies in the context of a large multicentre randomized one-season double-blind placebo-controlled dose-response study of subcutaneous alum-adsorbed grass pollen extract in patients with severe hay fever with or without seasonal asthma. Details of the clinical study and efficacy of treatment have previously been reported (20). Functional IgG-associated inhibitory activity was measured using assays of serum 'blocking' activity, as determined by the inhibition of FcεRII (CD23)-dependent binding of allergen–IgE complexes to B cells (11, 16) and inhibition of allergen–IgE interaction (15, 21). Moreover, differences in the clinical readout were investigated for patients subgrouped into three equal sizes based on their level of immunoreactive IgG₄ or IgG-associated inhibitory antibodies.

Methods

Treatment regime

An 8-month randomized double-blind placebo-controlled study of subcutaneous grass pollen immunotherapy (Alutard SQ[®], *Phleum pratense*; ALK-Abelló A/S, Hoersholm, Denmark) was conducted in 26 UK hospital clinics. The details of the immunotherapy protocol and clinical outcome measures, including symptoms and rescue medication scores, have been previously reported (20). A 1 : 1 : 2 randomization [placebo: active (10 000 SQ-U = 20 µg): active (100 000 SQ-U)] enabled a greater number of subjects in the 100 000 SQ-U-treated group to strengthen within-group comparison (see Table S1 in the Online Supporting information). Subcutaneous injections of standardized aluminium hydroxide-adsorbed extract of *P. pratense* (Alutard SQ grass pollen; ALK-Abelló) were administered. Up-dosing injections were given once a week for 6–8 weeks followed by monthly depot maintenance injections for 8 months. Maintenance injections for subjects treated with 100 000 and 10 000 standard quality units contained ~20 and ~2 µg of the major grass pollen allergen Phl p5, respectively. Maintenance injections were given every 6 ± 2 weeks. Study medication was provided as a suspension in vials. Placebo and active medications were identical, except for the omission of grass pollen extract from placebo injections. 100 000 SQ-U dose was prepared, and a direct tenfold dilution was performed to achieve 10 000 SQ-U.

Sera were collected before treatment (baseline visit; week 0), after up-dosing (1st maintenance injection visit; week 8),

during the peak season (seasonal visit; week 22) and at the end of study (final visit; week 32) from 221 patients (placebo $n = 55$; 10 000 SQ-U $n = 54$; and 100 000 SQ-U $n = 112$). Combined symptom and rescue medication scores were derived by the sum of the average symptom and medication score. The average daily symptom score and average daily medication score are calculated as the mean daily sum of symptoms or medication scores over the 3-week peak pollen season. Symptom scores were recorded for six parameters (four nasal and two eye). Each parameter was scored on a 4-point scale (0 = none, 1 = mild, 2 = moderate and 3 = severe). The four nasal symptoms were runny nose, blocked nose, sneezing and itchy nose. The two eye symptoms were gritty feeling/red/itchy eyes and watery eyes.

Immunological tests

Allergen-specific IgE and IgG₄ titres were measured directly in the serum samples, while the IgE-blocking factor reflects a competition between IgE and non-IgE antibodies for binding to allergen; inhibition of IgE-FAB reflects the effect of IgG antibodies on IgE-allergen complex formation and binding to CD23.

Phleum pratense-specific IgE antibody was measured by ADVIA Centaur sIgE assay (21). All samples were measured according to the manufacturer's instructions. Allergen-specific IgG₄ antibodies were measured by ELISA (enzyme-linked immunosorbent assay), with coating allergen concentration at 5 µg/ml; sera dilution at 1 : 100; and biotinylated anti-human IgG₄ antibody for detection at 1 : 100 000 (clone HP-6025;0 Sigma-Aldrich, Gillingham, UK). IgE-facilitated allergen binding (FAB) to B cells was performed as previously described (11, 16). Briefly, serum from a grass pollen-sensitized donor [specific IgE to rPhl p1 (34 ISAC units (ISU), rPhl p4 (13 ISU), rPhl p5 (124 ISU) and rPhl p6 (32 ISU)] was incubated with 1 µg/ml of *P. pratense* in the presence or absence of an equal volume of sera from study patients for 1 h at 37°C. EBV-transformed B cells (100 000 cells/test) were added for 1 h at 4°C and washed. Allergen-IgE complexes binding to B cells were detected using a fluorescein isothiocyanate-labelled polyclonal anti-IgE antibody (0.8 µg/ml; Dako-Cytomation Ltd, Ely, UK). Allergen-IgE binding to B cells was analysed by flow cytometry using WinMDI 2.8 software (J. Trotter, The Scripps Research Institute, La Jolla, CA, USA). IgE-blocking factor was measured by ADVIA Centaur Instrument as previously reported (15). Sera were tested with two different assay procedures: one according to the manufacturer's instructions for specific IgE measurements in serum samples (wash assay) and the other one excluded the wash step after catching IgE on the solid phase (no-wash assay). The no-wash assay allowed non-IgE antibodies to interact with the biotinylated allergens in competition with the solid-phase absorbed IgE antibodies (15). The numerical readout for the Centaur assay has previously been described (11, 15, 21). The assay readout is (IgE binding in competition with non-IgE)/(IgE binding with remaining Igs washed away). The ratio will be close to 1, that is equivalent to pretreatment values where no blocking antibodies have been induced.

Statistical analysis

Analysis of changes from pretreatment visit in log-transformed specific IgE, log-transformed specific IgG₄, IgE-FAB and specific IgE-blocking factor was performed with a linear mixed-effects model. The model included as fixed effects the pretreatment value, treatment group, visit and treatment-group-by-visit interaction. To allow correlation of observations on the same patient, a random subject effect was included. A different error variance was specified for each treatment group.

The relationship between combined symptom and medication scores during peak season and change in specific IgG₄ from pretreatment visit to 1st maintenance injection visit was analysed. The 221 subjects were allocated into three group levels of about equal size based on the quantity of specific IgG₄. In this way, an ordered categorical variable on three levels was created. Then, the combined symptom and medication score was analysed with a linear mixed-effects model, using the created categorical variable as fixed effect and the pollen region as a random effect. By including pollen region, possible differences in CSMS owing to differences in pollen exposure between different geographic regions in the UK are accounted for. Similar analyses were performed for the relationship between combined symptom and medication score during peak season and change in IgE-FAB and specific IgE-blocking factor. For the subgroup of 162 grass-allergic participants who received Alutard *P. pratense* extract 100 000 SQ-U or placebo, Friedman's proportion of treatment effect explained by IgG₄, IgE-FAB or specific IgE-blocking factor was estimated (22). Correlation coefficients were obtained using Spearman's rank method. All hypothesis testing was exploratory. Two-sided *P*-values are presented. *P*-values < 0.05 were considered statistically significant. All log transformations presented in this study are natural log transformations.

Results

Clinical results

The clinical results of this study have been reported previously (18). Briefly, symptom and medication scores in subjects treated with 100 000 SQ-U were significantly reduced when compared with those treated with placebo (29%, $P = 0.0001$; 32%, $P = 0.0007$, respectively). The 10 000 SQ-U-treated group showed reduced symptom scores (22%, $P = 0.013$) but no significant reduction in medication scores when compared with placebo-treated group (16%, $P = 0.16$, respectively). In this study, clinical results were expressed as a combined symptom and medication scores (see Table 1). During the pollen season (Fig. 1A), combined symptom and medication scores for the 100 000 SQ-treated group were significantly lower compared with those for placebo-treated group (32%, $P = 0.001$) (see Fig. 1B). Combined symptom and medication scores were numerically but not statistically significantly reduced (17%, $P = 0.15$) in subjects treated with 100 000 SQ compared with those of the 10 000 SQ-treated group. Similarly, a trend for reduced combined scores in the 10 000 SQ-treated group did not achieve significance compared with that of placebo-treated group (18%, $P = 0.11$).

Table 1 Combined symptom and medication scores during the pollen season

	Adjusted mean (SE)	Difference vs Placebo (95% CI), <i>P</i> -value	Difference of 100 000 SQ-U vs 10 000 SQ-U (95% CI), <i>P</i> -value
Combined symptom and medication scores whole season			
Alutard 100 000 SQ-U (<i>n</i> = 112)	5.84 (0.57)	-2.80 (-4.44, -1.15), <i>P</i> = 0.001	-1.21 (-2.86, 0.44), <i>P</i> = 0.15
Alutard 10 000 SQ-U (<i>n</i> = 54)	7.05 (0.76)	-1.58 (-3.50, 0.34), <i>P</i> = 0.11	
Placebo (<i>n</i> = 55)	8.63 (0.77)		
Combined symptom and medication scores peak season			
Alutard 100 000 SQ-U (<i>n</i> = 112)	7.94 (0.76)	-4.57 (-6.79, -2.35), <i>P</i> < 0.0001	-2.62 (-4.84, -0.40), <i>P</i> = 0.021
Alutard 10 000 SQ-U (<i>n</i> = 54)	10.6 (1.02)	-1.95 (-4.53, 0.63), <i>P</i> = 0.14	
Placebo (<i>n</i> = 55)	12.5 (1.03)		

Statistical comparison by means on ANOVA, including adjustment for pollen exposure.

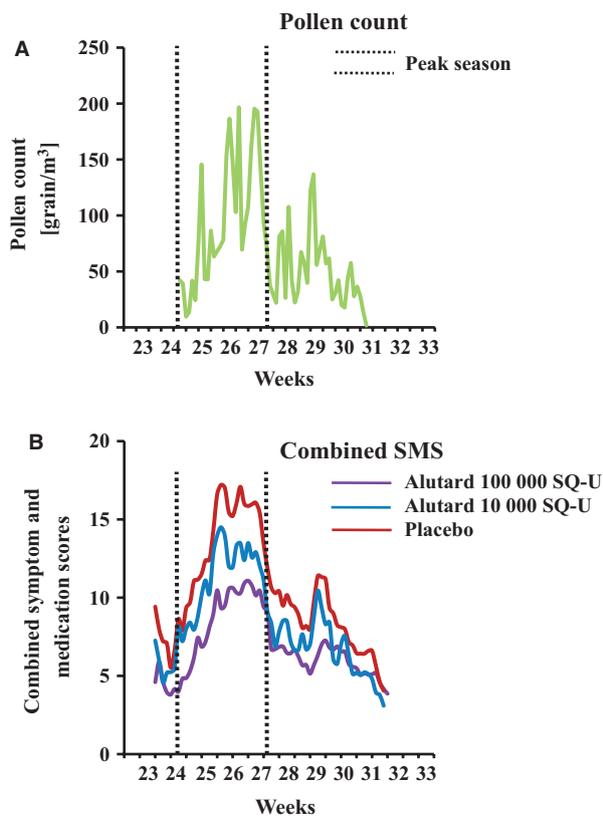


Figure 1 Subcutaneous grass pollen immunotherapy results in dose-dependent reduction in combined symptom and rescue medication scores. (A) Grass pollen counts (grain/m³). (B) Weekly average symptom and rescue medication scores in subjects treated with Alutard *Phleum pratense* 100 000 SQ-U (*n* = 112; purple line), 10 000 SQ-U (*n* = 54; blue line) and placebo (*n* = 55; red line).

Blunting of seasonal increases in specific IgE antibodies by immunotherapy

Across the whole pollen season, subjects treated with 100 000 SQ-U and 10 000 SQ-U had elevated levels of *P. pratense*-specific IgE antibodies at all time points (see Fig. 2). This increase was transient and followed by a significant decrease during the peak season when compared to 1st maintenance injection visit. In contrast, placebo-treated patients had reduced levels of IgE antibodies at the 1st maintenance injection visit compared to baseline followed by a steady increase

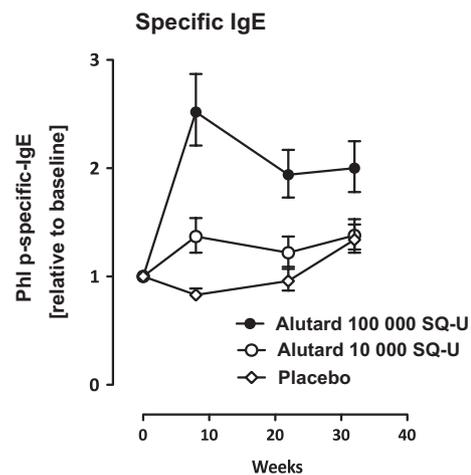


Figure 2 Subcutaneous allergen immunotherapy is associated with increases in *Phleum pratense*-specific IgE antibodies and blunting of seasonal increases in IgE antibodies. Data are shown as observed mean change (upper 95% confidence limit) in log-specific IgE from baseline.

Table 2 Change from baseline in Log-specific IgE antibodies, Log IgG₄ antibodies, relative IgE-facilitated allergen binding (FAB) and IgE-blocking factor in subjects treated with Alutard 100 000 SQ-U, 10 000 SQ-U or placebo

	1st maintenance injection week 8 adjusted mean (SE)	Seasonal visit week 22 adjusted mean (SE)	End of study week 32 adjusted mean (SE)
Log <i>Phleum pratense</i> -specific IgE antibodies (kU/ml)			
Alutard 100 000 SQ-U (<i>n</i> = 106, 105, 103)	1.08 (0.08)*, **	0.82 (0.08)*, **	0.83 (0.08)*, **
Alutard 10 000 SQ-U (<i>n</i> = 52, 51, 50)	0.49 (0.08)***	0.39 (0.08)***	0.50 (0.08)
Placebo (<i>n</i> = 54, 52, 55)	0.01 (0.08)	0.14 (0.08)	0.48 (0.08)
Log <i>P. pratense</i> -specific IgG ₄ antibodies (AU/ml)			
Alutard 100 000 SQ-U (<i>n</i> = 108, 105, 107)	1.28 (0.07)*, **	1.83 (0.08)*, **	2.16 (0.08)*, **
Alutard 10 000 SQ-U (<i>n</i> = 54, 52, 50)	0.67 (0.10)***	0.95 (0.10)***	0.96 (0.11)***
Placebo (<i>n</i> = 51, 51, 54)	0.02 (0.05)	-0.01 (0.05)	-0.02 (0.05)
FAB to B cells [%]			
Alutard 100 000 SQ-U (<i>n</i> = 108, 105, 107)	24.70 (1.79)*, **	41.58 (1.80)*, **	39.16 (1.79)*, **
Alutard 10 000 SQ-U (<i>n</i> = 54, 52, 50)	1.82 (2.62)***	8.47 (2.64)***	6.64 (2.67)***
Placebo (<i>n</i> = 54, 53, 55)	-6.64 (2.14)	-10.26 (2.15)	-11.26 (2.13)
IgE-blocking factor			
Alutard 100 000 SQ-U (<i>n</i> = 106, 105, 103)	0.37 (0.02)*, **	0.53 (0.02)*, **	0.54 (0.02)*, **
Alutard 10 000 SQ-U (<i>n</i> = 52, 51, 50)	0.09 (0.01)***	0.15 (0.01)***	0.14 (0.01)***
Placebo (<i>n</i> = 54, 52, 55)	-0.02 (0.01)	-0.03 (0.01)	-0.002 (0.007)

**P* < 0.0001 of statistical differences between 100 000 SQ-U- and placebo-treated groups within visits. Statistical differences between 100 000 SQ-U- and 10 000 SQ-U-treated groups within visits were determined.

***P* < 0.0001 of statistical differences between 10 000 SQ-U- and placebo-treated groups within visits were determined.

*** and *****P* < 0.0001 and *P* < 0.05, respectively.

in peak season and at the end of study (see Table 2). Specific IgE levels were raised in subjects treated with 100 000 SQ when compared to 10 000 SQ-treated group and placebo-treated patients. Specific IgE levels were also increased in the 10 000 SQ-treated group when compared to that in placebo-treated group.

Dose-dependent increases in *P. pratense*-specific IgG₄ antibodies following immunotherapy

Dose-dependent increases in serum grass pollen-specific IgG₄ antibody levels were evident throughout the study both within and between groups treated with 100 000 SQ and 10 000 SQ compared to baseline and when compared to placebo-treated patients (see Table 2 and Fig. 3A).

Dose-dependent increases in serum IgG-associated inhibitory activity following immunotherapy

Subjects treated with 100 000 SQ-U had significantly reduced allergen-IgE binding to B cells (see Fig. 3B) and increased IgE-blocking factor (see Fig. 3C) at all time points when compared to 10 000 SQ-U- and placebo-treated groups. Subjects treated with Alutard 10 000 SQ-U had reduced allergen-IgE binding to B cells at peak pollen season and at the end of the study visit when compared to placebo-treated group (see Table 2). Similarly, IgE-blocking factor was increased at all time points for this group when compared to placebo-treated patients.

Correlations between immunological measurements

Immunologic measurements were compared at the time of 1st maintenance injection. Significant correlations were observed between change from baseline in IgE-FAB and IgE-blocking factor (Spearman's correlation coefficient, *r* = 0.60, *P* < 0.0001) and between *P. pratense*-specific IgG₄ and specific IgE antibody concentrations (0.49, *P* < 0.0001). Despite this interrelationship, only functional antibody measurements (IgE-FAB) and IgE-blocking factor revealed a significant correlation with treatment effects.

IgG-associated serum inhibitory activity but not immunoreactive grass pollen-specific IgG₄ antibodies correlates with combined CSMS and RQoL

Serum inhibitory activity for IgE-FAB and IgE-blocking factor for all subjects at the 1st maintenance injection visit was correlated with individual combined symptom and medication scores during the subsequent pollen season. A modest but significant inverse correlation was observed for serum inhibitory activity for IgE-FAB (*r* = -0.25, *P* = 0.0002) and for IgE-blocking factor (*r* = -0.28; *P* < 0.0001) compared to combined symptom-rescue medication scores (see Fig. 4 A,B), whereas no significant correlation was observed for allergen-specific IgG₄ levels (*r* = -0.11, *P* = 0.12). Similarly, a significant inverse correlation was observed between serum inhibitory activity measured during the peak pollen season visit and combined symptom and medication scores (IgE-

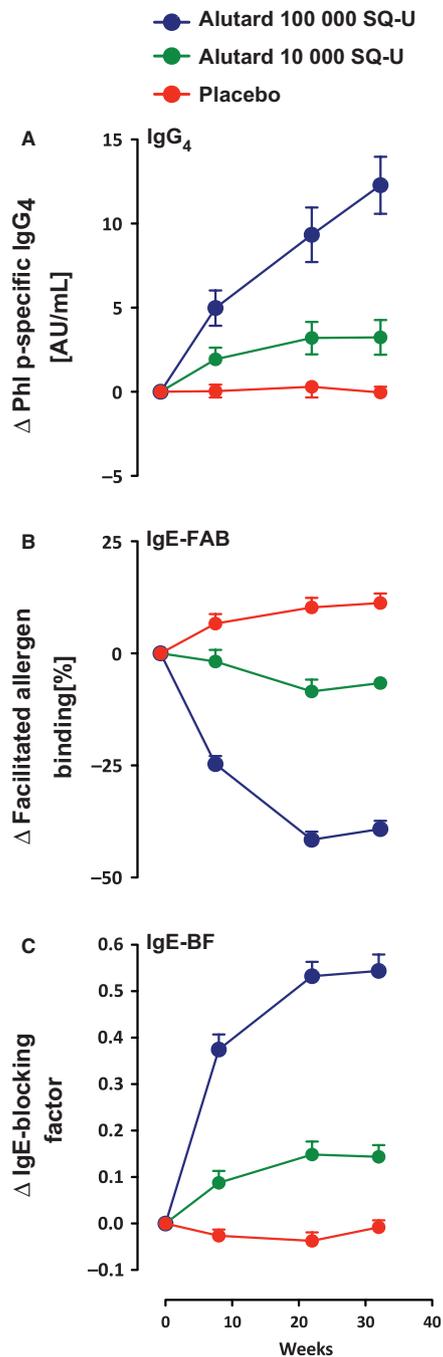


Figure 3 Time course and dose dependency of subcutaneous grass pollen immunotherapy-induced changes in allergen-specific IgG₄ antibodies and serum inhibitory activities. (A) *Phleum pratense*-specific IgG₄ antibody levels were measured by enzyme-linked immunosorbent assay. Data are shown as observed mean change in log concentration (upper 95% confidence limit). Serum inhibitory activity was measured by (B) IgE-FAB assay and (C) ADVIA Centaur automated analyser measurement of IgE levels that included a 'no-wash' step to measure IgE-blocking factor. Data are shown as observed mean change (upper 95% confidence limit) in percentage relative facilitated allergen binding and IgE-blocking factor from baseline.

FAB, $r = -0.27$, $P < 0.0001$; IgE-blocking factor, $r = -0.30$, $P < 0.0001$). Furthermore, the relationship between serum inhibitory activities (as opposed to IgG₄ levels) and clinical response was confirmed in all treated subjects that received Alutard 100 000 SQ-U and 10 000 SQ-U when placebo-treated patients were not included in the analysis (see Table S2 in the Online Supporting information). In contrast, no significant correlation was observed when the analysis was confined to the 100 000 SQ-treated group, possibly reflecting the consequent reduced numbers included in the analysis or possibly a less heterogeneous response in this group.

A modest but significant inverse correlation was observed at 1st maintenance visit between RQoL scores and serum inhibitory activity (IgE-FAB, $r = -0.17$, $P = 0.01$; IgE-blocking factor, $r = -0.15$, $P = 0.03$) but not with grass pollen-specific IgG₄ antibody levels ($r = -0.10$, $P = 1.26$).

For the third of subjects with the highest change from pre-treatment visit to 1st maintenance visit in IgE-FAB, compared to the third with the lowest change, the difference in combined symptom and medication score was statistically significant ($P < 0.0001$) (see Table 3). A similar result was found for IgE-blocking factor ($P < 0.0005$) as opposed to IgG₄ ($P = 0.59$). For the subgroup of subjects treated with 100 000 SQ-U or placebo, the estimated treatment effect of Alutard 100 000 SQ-U on combined symptom and medication scores over peak season was -4.45 (6.84; -2.06). This treatment effect was altered by 40% when adjusted for change in IgE-IgE-FAB -2.69 (-6.01 ; 0.62), 33% when adjusted for change in IgE-blocking factor -2.97 (-7.85 ; 1.91) and 13% when adjusted for change in IgG₄ -3.88 (-7.95 ; 0.20).

Discussion

Allergen immunotherapy resulted in time- and dose-dependent increases in antibody-associated serum inhibitory activity for IgE-FAB and increases in IgE-blocking factor. A significant inverse correlation was observed between serum inhibitory activity identified immediately after up-dosing (at the 1st maintenance injection) and subsequent combined symptom-rescue medication scores during the pollen season. In contrast, this was not observed for immunotherapy-induced increases in grass pollen-specific IgG₄ antibodies. Changes in functional allergen-specific antibodies accounted for up to 40% of the treatment effect, whereas changes in specific IgG₄ accounted for only 13%. Although further studies that include clinical data from a baseline observational assays are needed, these findings suggest that functional assays of postimmunotherapy serum inhibitory activity may be more predictive of the clinical response to immunotherapy than serum allergen-specific IgG₄ levels.

Previous studies have demonstrated that IgE-dependent CD23-mediated allergen presentation occurs in atopic individuals and requires 100- to 1000-fold lower allergen concentrations to activate allergen-specific T-helper cells, while postimmunotherapy serum contains IgG-associated inhibitory activity that blocks this activity (23, 24). We have confirmed these findings in a dose- and time-dependent fashion,

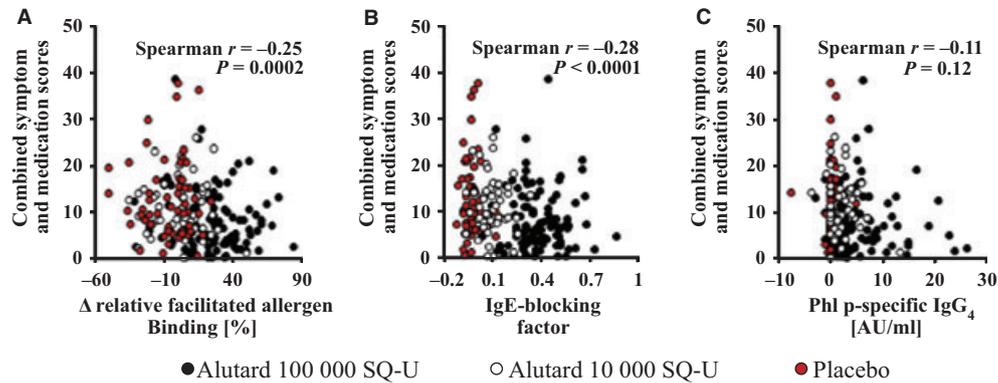


Figure 4. Postimmunotherapy serum inhibitory activity but not IgG₄ levels measured at 1st maintenance injection visit correlates with seasonal combined symptom and rescue medication scores. Correlation of combined daily symptom and medication scores over the 3-week peak season with change from baseline of (A) facilitated allergen binding, (B) IgE-blocking factor and (C) *Phleum pratense*-specific IgG₄ antibody levels at 1st maintenance (week 8 of treatment). Correlations were obtained using Spearman's rank method.

Table 3 The relationship between changes in specific IgG₄, IgE-facilitated allergen binding (FAB) and IgE-blocking factor at first maintenance injection visit and subsequent peak seasonal combined symptom and medication scores

	N	Combined symptom and medication score adjusted mean (SE)	Difference vs level 3, (95% CI), P-value
<i>Phleum pratense</i> -specific IgG ₄ antibodies (AU/ml)			
Level 1 (2.9, 26.1)	71	9.4 (0.98)	-0.62 (-2.89, 1.64), P = 0.59
Level 2 (0.2, 2.9)	71	9.96 (1.00)	-0.06 (-2.34, 2.21), P = 0.96
Level 3 (-7.6, 0.1)	71	10.0 (0.99)	
FAB to B cells (%)			
Level 1 (-21, -84)	72	7.6 (0.99)	-4.50 (-6.73, -2.27), P < 0.0001
Level 2 (-2, -20)	72	10.3 (1.00)	-1.78 (-4.01, 0.46), P = 0.12
Level 3 (50, -2)	72	12.1 (1.01)	
IgE-blocking factor			
Level 1 (0.3, 1)	72	8.35 (1.01)	-4.06 (-6.31, -1.81), P = 0.0005
Level 2 (0.06, 0.3)	68	9.29 (1.03)	-3.12 (-5.40, -0.84), P = 0.0076
Level 3 (-0.2, 0.06)	72	12.4 (1.02)	

The levels were defined by subdividing the subjects according to their value into three groups about equal size. Estimates are obtained from the mixed-model ANOVA including all available data from all three groups for each pairwise comparison. The model includes as fixed effect the factor level and as random effect the pollen region. Two-sided P-values are presented.

although the changes in serum inhibitory activity measured in subjects treated with the lower dose of Alutard *P. pratense* (10 000 SQ-U) were significant only at peak season and not at pre-season (following only 8 weeks of treatment) as observed for the high-dose group. These data suggest that to induce inhibitory antibodies, an allergen-dose threshold needs to be reached. Moreover, the data support previous studies (6, 9, 11, 15, 25) demonstrating that these antibodies inhibit allergen-IgE interaction and prevent CD23-mediated IgE-facilitated allergen presentation as well as effector cell activation. Additionally, we have shown that a correlation exists between serum IgG-associated inhibitory activity and clinical outcomes following immunotherapy, which is not apparent for allergen-specific IgG₄ levels. Interestingly, these correlations could be demonstrated shortly after up-dosing, suggesting that the effect of immunotherapy might predict

maintained efficacy for the following season on the basis of IgE-FAB or IgE-blocking factor measurements. Even better correlations between serum inhibitory activity and clinical response to treatment may be obtained by including baseline clinical data obtained during a preceding observational pollen season and by measuring serum inhibitory activity to individual major allergens (as opposed to the allergen extract).

In a subanalysis of $n = 20$ for all three group (placebo, 10 000 SQ-U and 100 000 SQ-U) at all time points (week 0, 8, 22 and 32), we observed time- and dose-dependent increases in *P. pratense*-specific IgG and IgG₁ antibodies in agreement with previous findings (3). The functional role of allergen-specific inhibitory IgG₁ and IgG₄ antibodies has been demonstrated in other studies. Using monoclonal Bet v 1-specific IgG₁ and polyclonal IgG₁ and IgG₄ antibodies,

these antibodies inhibited basophil histamine release in SIT-treated patients (9, 26, 27). The possible mechanisms of how grass pollen-specific IgG₁ and IgG₄ antibody subclasses contribute towards desensitization are not very well understood. However, there is now ample evidence to support that these antibodies inhibit allergen-specific IgE interaction and prevent CD23-mediated IgE-facilitated allergen presentation (10, 11, 15, 25).

It can be speculated that the distinct immunological characteristics of IgG₄ (monovalent/bispecific, low affinity for certain Fc γ receptors, lack of complement activation, and binding to the inhibitory Fc γ RIIB) may make this IgG subclass more effective in inhibiting IgE-mediated reactions (12, 28–31). However, increases in IgG₄ per se showed no correlation with clinical outcomes when measured after up-dosing. In line with this observation, a previous study did not support a role for the low-affinity IgG receptor Fc γ RIIB in mediating postimmunotherapy serum inhibitory activity for specific allergen-induced basophil histamine release (32).

IgG₄ antibodies were measured by *in house* solid-phase ELISA, which is semi-quantitative, reported as arbitrary units/millilitre (AU/ml) and not standardized against a reference preparation. The possible technical disadvantages for using ELISA may be the measurements of both high- and low-affinity IgG₄ antibodies against the allergen extract (*P. pratense*). Moreover, it is likely that only specific IgG₄ antibodies with high affinity and avidity are functionally relevant. It is likely that measuring specific IgG₄ to the major allergens, Phl p 1 and Phl p 5, may have shown an improved correlation. In this study, Phl p extract showed optimal allergen–IgE binding (max binding 60%) to B cells in the FAB assay, while Phl p 1 and Phl p 5 showed <20% and <40% allergen–IgE binding, respectively. Therefore *P. pratense* extract was used in the FAB assay and for measuring specific IgG₄.

A close correlation was demonstrated between FAP with autologous serum and FAP with standard/heterologous serum (15), indicating that the effect of blocking antibodies is not directly dependent on the individual patients' IgE repertoire. In contrast, clear differences were seen when recombinant IgG was used to block complex formation induced with two or three recombinant IgE antibodies (33). However, the complex mixture of IgE antibodies in autologous serum competing with the complex mixture of SIT-induced IgG in the same serum sample may lead to comparable values for autologous and standard serum analyses. This is also reflected in the current study by the close correlation between FAB (heterologous IgE) and IgE-blocking factor (autologous IgE).

When evaluating the importance of the IgG₄ antibody subclass, it is important to emphasize that the concentration of allergen-specific IgG₄ is a small proportion of the total IgG concentration and that measurement of immunoreactive IgG₄ levels does not account for the affinity of these antibodies or the relevance of their specificity for competition with IgE–allergen binding. In this context, molecular characterization of allergen-specific IgG₄ antibodies revealed that the concentration, epitope specificity and antibody affinity are all rele-

vant for optimal inhibition (34). The overall avidity of high- and low-affinity allergen-specific IgG₄ antibodies rather than a single high-affinity Bet v 1-specific IgG₄ antibody clone as shown by Flicker and colleagues may be proven important in blocking interaction of allergen-specific IgE antibodies with high- and low-affinity IgE receptors. In general, this may explain why functional assays reflecting the competition between IgE and non-IgE antibodies for allergen binding seem to reflect the clinical effect more closely.

Furthermore, induction of regulatory T-cell activity may also contribute to the production of allergen-specific IgG₄ through IL-10 production and IgA through TGF- β production (35). This may in turn, through inhibition of CD23-mediated IgE-facilitated allergen presentation, lead to a change in the activation threshold for the allergen-specific Th2 cells and to T-cell activation through the presentation of the allergen by professional APC such as DC or macrophages, which requires higher allergen concentrations and favours Th1 development.

In summary, successful grass pollen subcutaneous immunotherapy was associated with dose- and time-dependent changes in immunoreactive and functional IgG antibody responses. A significant correlation was observed between serum inhibitory antibodies and subsequent seasonal combined symptom and rescue medication scores, whereas this was not seen for immunoreactive IgG₄ levels as measured by ELISA. These findings suggest that serum inhibitory antibodies may possibly be useful surrogate markers of the clinical response to immunotherapy. However, to further evaluate the role of serum inhibitory activity in predicting the individual response to immunotherapy, further studies that include antibody measurements and clinical outcomes recorded during a baseline pollen season for comparison with those after immunotherapy would be required.

Acknowledgments

This study was supported by grants from the Immune Tolerance Network, National Institutes of Health, USA, a Biotechnology and Biological Sciences Research Council UK (BBSRC) studentship case award and ALK-Abelló, Hørsholm, Denmark.

Author contributions

S. R. Durham, P. A. Wurtzen and K. Lund participated in designing research hypotheses. M. H. Shamji performed the experimental work, analysed data, interpreted results and wrote the manuscript. H. Ipsen performed the IgE-blocking factor analyses. J. N. Francis participated in the initial design of the study. S. R. Durham, M. Calderon and A. J. Frew were investigators in the clinical trial. C. Ljørring performed statistical analyses. P. A. Wurtzen, C. Ljørring and I. Kimber participated in discussions of data analysis and interpretation and contributed to manuscript preparation. S. R. Durham advised and critically reviewed the manuscript. The manuscript was finalized by M. H. Shamji with the assistance of all authors. M. Larché participated in the original hypoth-

esis, and in the design of the experimental work in this manuscript.

Conflict of interest

S. R. Durham is a member of the Immune Tolerance Network Steering Committee, has consulting arrangements with ALK-Abelló and has received research support from ALK-Abelló. C. Ljørring, H. Ipsen, K. Lund and P. A. Würtzen are employed by ALK-Abelló and hold stocks in the company. A. J. Frew has consulting arrangements with ALK-Abelló and has received research support from ALK-Abelló. The rest of the authors have declared that they have no conflict of interest.

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Supporting Information

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Table S1. Subject characterization.

Table S2. Correlation of serum inhibitory activity and grass pollen-specific IgG₄ antibodies levels measured at 1st maintenance injection and peak season visits with seasonal combined symptom and medication scores.

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