

# Long-term tolerance after allergen immunotherapy is accompanied by selective persistence of blocking antibodies

Louisa K. James, PhD,<sup>a\*</sup> Mohamed H. Shamji, PhD,<sup>a\*</sup> Samantha M. Walker, PhD, RGN,<sup>a</sup> Duncan R. Wilson, MD, FRCP,<sup>a</sup> Petra A. Wachholz, PhD,<sup>a</sup> James N. Francis, PhD,<sup>a</sup> Mikila R. Jacobson, PhD,<sup>a</sup> Ian Kimber, PhD,<sup>b</sup> Stephen J. Till, PhD, MRCP,<sup>a</sup> and Stephen R. Durham, MD, FRCP<sup>a</sup> *London and Manchester, United Kingdom*

**Background:** Grass pollen immunotherapy for allergic rhinitis is a disease-modifying treatment that results in long-term clinical tolerance lasting years after treatment discontinuation. Active treatment is associated with generation of inhibitory grass pollen-specific IgG antibodies capable of blocking allergen-IgE interactions.

**Objectives:** We sought to investigate the involvement of IgG-associated inhibitory antibodies with long-term clinical tolerance after discontinuation of grass pollen immunotherapy. **Methods:** We conducted a 4-year study in which patients who had moderate-to-severe allergic rhinitis underwent a randomized, double-blind, placebo-controlled discontinuation of subcutaneous grass pollen immunotherapy. All subjects received grass pollen immunotherapy injections for 2 years ( $n = 13$ ), followed by a further 2 years of either active ( $n = 7$ ) or placebo ( $n = 6$ ) injections. Clinical outcomes included seasonal symptoms and use of rescue medication. Serum specimens were collected at baseline and after 2 and 4 years for quantification of allergen-specific IgG antibodies. Sera were also tested for IgG-dependent inhibitory bioactivity against IgE-allergen binding in cellular assays by using flow cytometry and confocal microscopy to detect binding of IgE-grass pollen allergen complexes to B cells. **Results:** Clinical improvement was maintained after 2 years of discontinuation. Although immunotherapy-induced grass pollen-specific IgG1 and IgG4 levels decreased to near-preimmunotherapy levels during discontinuation, inhibitory bioactivity of allergen-specific IgG antibodies was maintained unchanged.

**Conclusion:** Grass pollen immunotherapy induces a subpopulation of allergen-specific IgG antibodies with potent inhibitory activity against IgE that persists after treatment discontinuation and that could account for long-term clinical tolerance. (*J Allergy Clin Immunol* 2011;127:509-16.)

**Key words:** Immunotherapy, IgE, IgG4, facilitated antigen presentation, CD23

Grass pollen allergy affects up to a quarter of the population in many Western countries and represents a major economic, societal, and health burden.<sup>1-3</sup> In general, avoidance of exposure to grass pollen is not possible during the summer months. Although pharmacotherapy is often effective, almost half of patients show a poor response to conventional treatments.<sup>4,5</sup> In these subjects grass pollen-specific immunotherapy (desensitization) can be highly effective.<sup>6,7</sup> A remarkable feature of immunotherapy is the induction of long-term antigen-specific tolerance, namely persisting clinical desensitization to grass pollen even after immunotherapy is discontinued. We previously showed that 3 or 4 years of grass pollen immunotherapy results in continuing clinical remission and reduced responsiveness in allergen challenge tests for at least 3 years after placebo-controlled discontinuation.<sup>8</sup>

Grass pollen immunotherapy induces vigorous allergen-specific IgG antibody responses within 2 to 3 months of starting injections, with levels of the IgG4 isotype showing the greatest proportionate increase.<sup>9</sup> These antibodies directly inhibit IgE-dependent histamine release and antigen presentation to T cells mediated through high-affinity (Fc $\epsilon$ RI) and low-affinity (Fc $\epsilon$ RII) receptors, respectively.<sup>10,11</sup> This inhibition probably occurs through direct competition for IgE-allergen binding by IgG isotypes. Antibodies, especially IgG4, are likely produced by B cells under the influence of allergen-specific regulatory T cells,<sup>12-14</sup> which appear early during immunotherapy.<sup>9</sup> The mechanisms associated with long-term clinical tolerance after immunotherapy discontinuation treatment are unknown.

We tested the hypothesis that persistence of functional allergen-specific IgG antibodies is responsible for long-term clinical tolerance after immunotherapy is discontinued. In a placebo-controlled study of grass pollen immunotherapy discontinuation, we examined clinical outcomes, allergen-specific IgE and IgG levels, and measurements of inhibitory serum IgG-dependent bioactivity against IgE in validated assays. We report that allergen-specific IgG1 and IgG4 levels decrease to near-preimmunotherapy baseline levels after 2 years of vaccine discontinuation. However, the overall functional inhibitory activity against IgE mediated by the residual IgG is almost

From <sup>a</sup>Allergy 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, and <sup>b</sup>the Faculty of Life Sciences, University of Manchester.

\*These authors contributed equally to this work.

Supported by grants from the Immune Tolerance Network (S.R.D.), Asthma UK (S.R.D. and S.J.T.), the Health Foundation (S.J.T.), and the London Law Trust (L.K.J.), and a Biotechnology and Biological Sciences Research Council (BBSRC) studentship case award (M.H.S.).

Disclosure of potential conflict of interest: S. M. Walker has received consulting fees and research support from ALK-Abelló. S. R. Durham has received lecture fees and research support from ALK-Abelló and has consulted for ALK-Abelló, Circassia, and Greer Laboratories. The rest of the authors have declared that they have no conflict of interest.

Received for publication August 18, 2010; revised December 17, 2010; accepted for publication December 17, 2010.

Reprint requests: Stephen R. Durham, MD, FRCP, Allergy and Clinical Immunology, National Heart and Lung Institute, Faculty of Medicine, Imperial College London, Sir Alexander Fleming Building, London, SW7 2AZ, United Kingdom. E-mail: s.durham@imperial.ac.uk.

0091-6749/\$36.00

© 2011 American Academy of Allergy, Asthma & Immunology

doi:10.1016/j.jaci.2010.12.1080

**Abbreviations used**

FAB: Facilitated allergen binding  
IQR: Interquartile range

completely maintained, suggesting a possible mechanism for long-term tolerance to grass pollen.

**METHODS****Subjects**

All subjects were selected on the basis of moderate-to-severe seasonal allergic rhinitis and poor symptom control in previous years despite regular medication use. Subjects had a positive skin prick test response (wheal >5 mm) to *Phleum pratense* grass pollen extract (ALK Soluprick; ALK-Abelló, Hørsholm, Denmark). Patients were excluded if they had a clinical history of other allergies, chronic asthma, or another significant medical illness. Patients with positive skin test responses to other allergens in the absence of symptoms on exposure to the relevant allergen or allergens were not excluded. Patients with mild seasonal asthma were included, provided their symptoms were adequately controlled with inhaled  $\beta_2$ -adrenergic agonist bronchodilators. The study protocol was approved by the Royal Brompton and Harefield Hospitals NHS Trust Ethics Committee. All subjects provided written informed consent.

**Study design**

Forty-four subjects were recruited before the pollen season, and clinical parameters were monitored during a baseline season. Twenty-two subjects were randomized to receive Alutard SQ grass pollen extract according to a modified cluster regimen of injections, as previously described.<sup>15</sup> Briefly, participants attended twice weekly for 4 weeks for up dosing injections followed by monthly maintenance injections for a 2-year period at a dose of 100,000 standard quality units of *P pratense* adsorbed with aluminum hydroxide, which is equivalent to 20  $\mu$ g of major allergen (Phl p 5). Placebo-treated subjects (n = 22) received injections containing 0.01  $\mu$ g/mL histamine. Thirteen of the 22 participants who had received active therapy then consented to take part in a randomized controlled trial of immunotherapy discontinuation (Fig 1). There was no difference in seasonal symptom scores during year 2 for those 13 subjects who consented to continue to the double-blind withdrawal phase compared with the total 22 participants who received active immunotherapy during years 1 and 2. Participants were randomized in double-blind fashion to continue active immunotherapy (n = 7) or to receive placebo injections (n = 6) for a further 2 years. The 2 groups were well matched at baseline (Table I). Subjects kept diary cards to record symptom scores and medication use for each pollen season throughout the study.<sup>15</sup> Individual symptoms in the nose, eye, throat, and chest were each scored daily on a scale of 0 to 3 (none, mild, moderate, and severe, respectively). Each use of rescue medication was recorded daily and scored as either 1 (per use of each sodium cromoglycate eye drop or sodium cromoglycate nasal spray or each salbutamol inhalation from a metered-dose inhaler) or 2 (per use of each short-acting antihistamine tablet [acrivastine, 8 mg] or 5-mg prednisolone tablet).

**Immunologic tests**

The antibodies used in this study are described in the Methods section of this article's Online Repository at [www.jacionline.org](http://www.jacionline.org). Serum total and specific IgE levels were measured by using an immunoenzymatic assay (ImmunoCAP; Phadia AB, Uppsala, Sweden). Serum allergen-specific IgG4 antibodies were measured by using an ELISA with 5  $\mu$ g/mL *P pratense* extract (ALK-Abelló) for capture.<sup>9</sup> The IgE-dependent facilitated allergen binding (IgE-FAB) assay was performed, as previously described,<sup>16</sup> to assess the inhibitory bioactivity of sera against allergen-IgE complex formation. Briefly, allergen-IgE complexes were formed by incubating 20  $\mu$ L of sera containing high concentrations of *P pratense*-specific IgE (>100 IU/mL) with 1  $\mu$ g/mL *P pratense*

extract in the presence or absence of 20  $\mu$ L of sera (taken from participants before and at intervals after immunotherapy) for 1 hour at 37°C. Complexes were then incubated with 100,000 EBV-transformed human B cells for 1 hour on ice, and binding of complexes to B cells was quantified either by means of flow cytometry (FACSCalibur flow cytometer; Becton Dickinson, Franklin Lakes, NJ) or confocal laser scanning microscopy (Zeiss LSM-510; Carl Zeiss, Thornwood, NY). Surface-bound IgE-allergen complexes were detected either directly with a fluorescently conjugated anti-IgE antibody or indirectly by staining with a fluorescently conjugated anti-CD23 antibody and observing IgE-allergen complex-dependent surface CD23 aggregation ("beading," Fig 2 and see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Further methodological details are available in the Methods section of this article's Online Repository.

To determine whether the contribution of IgG4 to inhibitory bioactivity was affected by immunotherapy discontinuation, IgG4 was depleted from serum samples by means of passage over an anti-IgG4 Sepharose column (MH164-4; CLB, Amsterdam, The Netherlands), with depletion confirmed by using an ELISA for allergen-specific IgG4 and nephelometry (BN-ProSpec Nephelometer; Dade Behring, Marburg, Germany) for total IgG4 (see Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Inhibitory bioactivity against IgE-allergen complex formation and binding to B cells was also tested by using freshly isolated B cells from allergic subjects in place of CD23-enriched EBV-transformed B cells. For this purpose, PBMCs were isolated by means of density gradient centrifugation (Histopaque-1077; Sigma-Aldrich, St Louis, Mo) of whole blood, and B cells were isolated by means of negative selection with magnetically labeled microbeads (MACSTM B Cell Isolation Kit II; Miltenyi Biotech, Auburn, Calif).

**Statistics**

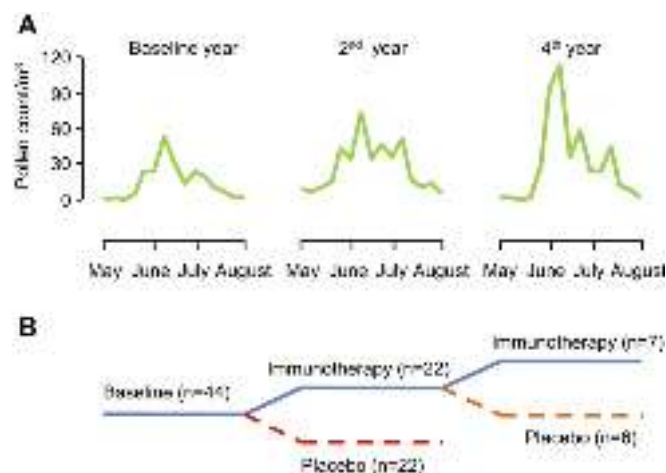
Between-group comparisons were performed with the Mann-Whitney *U* test, and within-group comparisons were performed with the Wilcoxon matched-pairs signed-rank test. Correlations between symptom and medication scores and sera inhibitory activity were assessed by calculating the Spearman rank correlation coefficient. The statistical software package used was GraphPad Prism, version 4 (GraphPad Software, Inc, La Jolla, Calif). *P* values of less than 5% were considered significant.

**RESULTS****Persistence of clinical benefit 2 years after discontinuation of grass pollen immunotherapy**

Thirteen patients who had received 2 years of treatment as part of a clinical trial<sup>15</sup> underwent randomized, double-blind discontinuation of therapy to investigate the mechanisms associated with long-term tolerance after grass pollen immunotherapy (Fig 1, B). The patients who underwent placebo-controlled discontinuation (n = 6) were well matched with those who continued to receive active immunotherapy (n = 7) for age, sex, wheal size in response to grass pollen skin prick testing at enrollment, baseline visual analog symptom scores, and baseline bronchial hyperreactivity to methacholine (PC<sub>20</sub>; Table I). Combined rhinitis symptom and medication scores for both the maintenance and discontinuation groups were temporally related to pollen counts (Fig 1, A), remained low, and were similar to those recorded during the preceding 3 years (Fig 3, A). Thus clinical tolerance was maintained after the 2 years of immunotherapy discontinuation.

**Immunoreactive grass pollen-specific antibodies after discontinuation of treatment**

Sera collected after 2 years of immunotherapy revealed increases in the levels of grass pollen-specific IgG1 (Fig 3, C) and IgG4 (Fig 3, D) in both those patients who subsequently



**FIG 1.** Study design and pollen counts. **A**, Weekly average grass pollen counts. **B**, Forty-four patients with hay fever were monitored for 1 pollen season and then randomized to receive subcutaneous allergen-specific immunotherapy or placebo injections for 2 years. Thirteen patients who received active treatment consented to a further 2-year, randomized, double-blind discontinuation of immunotherapy.

went on to receive a further 2 years' immunotherapy (IgG1, median 13-fold increase: interquartile range [IQR], 7.0-76.0 [ $P = .03$ ]; IgG4, median 173-fold increase: IQR, 6.4-203.0 [ $P = .03$ ]) and those who underwent placebo-controlled discontinuation (IgG1, median 56-fold increase: IQR, 21.5-179.2 [ $P = .03$ ]; IgG4, median 76-fold increase: IQR, 28.5-151.4 [ $P = .03$ ]).

In contrast, titers of both grass pollen-specific IgG subclasses were dissociated from clinical outcomes after immunotherapy discontinuation. After 2 years of discontinuation, grass pollen-specific IgG4 levels decreased from their peak (median change, 81%; IQR, 76.2-86.1 [ $P = .03$ ]) in contrast to the levels in patients who continued with immunotherapy, which were mostly unchanged (median, 3.7%; IQR, -65.9 to 38.8 [ $P = .9$ ]; Fig 3, D). Despite the marked decrease in allergen-specific IgG4 levels during discontinuation, the levels still remained higher than baseline preimmunotherapy levels ( $P = .03$ ). A similar pattern of responses was observed for IgG1 (Fig 3, C).

Subjects were matched for total and allergen-specific IgE levels (Table I) at baseline. IgE levels did not change significantly at 2 years for either group, but allergen-specific IgE levels decreased at 4 years after continued immunotherapy ( $P < .03$ ), whereas in the withdrawal group IgE levels remained unchanged (Table II).

### Inhibitory activity of IgG antibodies persists after stopping immunotherapy

The inhibitory bioactivity of sera was examined by measuring its ability to block IgE-grass pollen complex binding in cellular assays.<sup>16</sup> Blocking of IgE-grass pollen allergen complex binding to the low-affinity IgE receptor CD23 (FcεRII) on the surface of EBV-transformed B cells as surrogate antigen-presenting cells was quantified by means of either flow cytometry or confocal microscopy. In all patients tested sera collected after 2 years of immunotherapy inhibited binding of IgE-allergen complexes to B cells, as detected both by means of flow cytometry (Fig 3, B and E) and confocal microscopy (Fig 4, A and B), when compared with baseline serum collected before starting

**TABLE I.** Baseline demographic data

	4-y IT (n = 7), median (IQR)	2-y IT + 2-y placebo (n = 6), median (IQR)
Age (y)	33 (32-36)	35 (30-37)
Sex (male/female)	4/3	4/2
Skin prick test (mm <sup>2</sup> )	49.0 (25.0-61.0)	50.0 (45.0-58.0)
Allergen-specific IgE (Phl p 1 and Phl p 5; kU/L)	26.6 (19.2-56.4)	31.9 (8.7-100)
Total IgE (kU/L)	133.5 (23.2-320.8)	375.5 (118.3-734.0)
Visual analog score	3.75 (1.60-5.80)	3.35 (3.08-3.63)
Methacholine PC <sub>20</sub>	6.0 (3.2-15.0)	3.75 (2.63-13.2)

Subjects who received 4 years of immunotherapy were matched at baseline with those who received 2 years of immunotherapy plus 2 years of placebo for age, sex, grass pollen skin test response size, symptom scores, and levels of airway methacholine responsiveness.

IT, Immunotherapy.

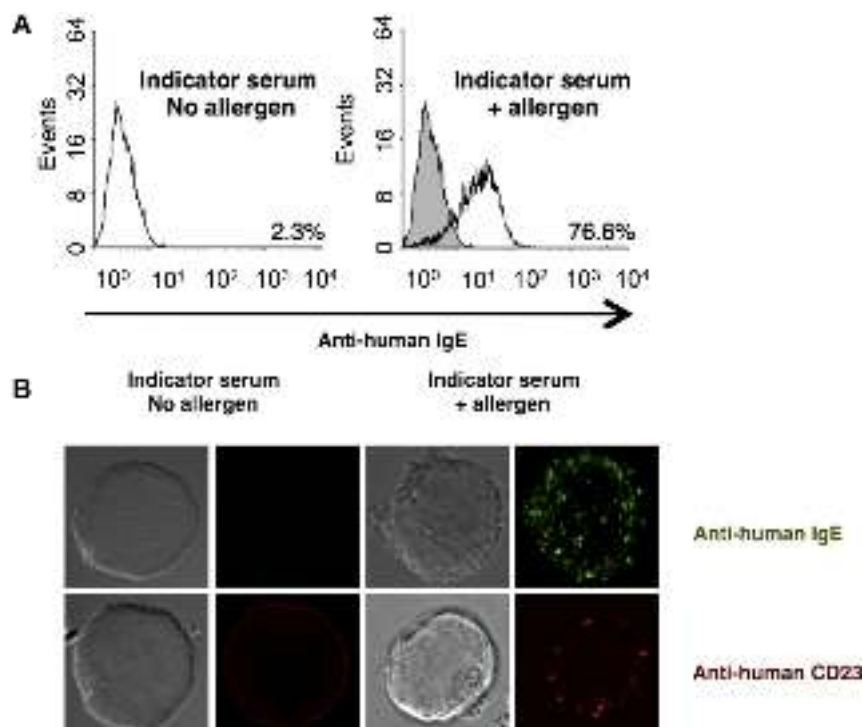
immunotherapy ( $P = .03$  in all cases). In the case of confocal microscopy, abrogation of IgE-grass pollen allergen complex binding to B cells by sera from patients receiving immunotherapy was also associated with disruption of surface CD23 aggregates (ie, beading) and restoration of a linear homogenous distribution (Fig 4, C and D). Inhibitory activity of sera from patients receiving immunotherapy could also be demonstrated in assays by using freshly isolated B cells in place of CD23-enriched EBV-transformed B cells, confirming that the latter are representative of untransformed native B cells (see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

Next, the inhibitory activity of sera after immunotherapy discontinuation was examined. At the end of year 4 after the placebo-controlled 2-year discontinuation phase, changes in serum inhibitory activity did not significantly differ between patients who continued or discontinued the therapy (Figs 3, B and E, and 4, A and B). Thus clinical tolerance paralleled maintenance of serologic inhibitory activity and was clearly dissociated from the absolute levels of grass pollen-specific IgG1 and IgG4 (Fig 3, C and D). Analyzing all patients at the end of the study, the magnitude of inhibition of allergen-IgE binding to B cells correlated inversely with combined symptom and medication scores (Spearman coefficient of correlation:  $r = -0.65$ ,  $P = .02$ ).

Allergen-specific IgG4 antibodies have been shown to account for the majority of inhibitory activity for IgE-FAB in patients receiving allergen immunotherapy.<sup>17</sup> We therefore examined the contribution of IgG4 to the persistent serologic inhibitory activity after discontinuation by performing bioassays with sera depleted of IgG4 (Fig 5). Depletion of IgG4 from sera both before and after discontinuation of immunotherapy resulted in a reduction in blocking activity, the effect being even greater ( $P = .03$ ) in patients after discontinuation.

### DISCUSSION

We have shown, under double-blind conditions, that long-term clinical tolerance after grass pollen immunotherapy is associated with persistence of potent serum inhibitory activity against IgE-allergen binding. After 2 years of discontinuation, during which patients remained in clinical remission, inhibitory activity in cellular assays dependent on IgE-grass pollen binding was maintained unchanged. At that time, 38% of this activity was attributable to IgG4, despite titers of serum immunoreactive grass pollen-specific IgG4 decreasing by 79% during discontinuation.



**FIG 2.** CD23-mediated IgE-FAB. **A** and **B**, Allergen-IgE complexes bound to EBV-transformed B cells were detected by means of flow cytometry (Fig 2, **A**) and laser scanning microscopy (Fig 2, **B**). **C**, CD23 receptor coaggregation was visualized in the presence or absence of grass pollen allergen. Data shown are representative results from 3 independent experiments.

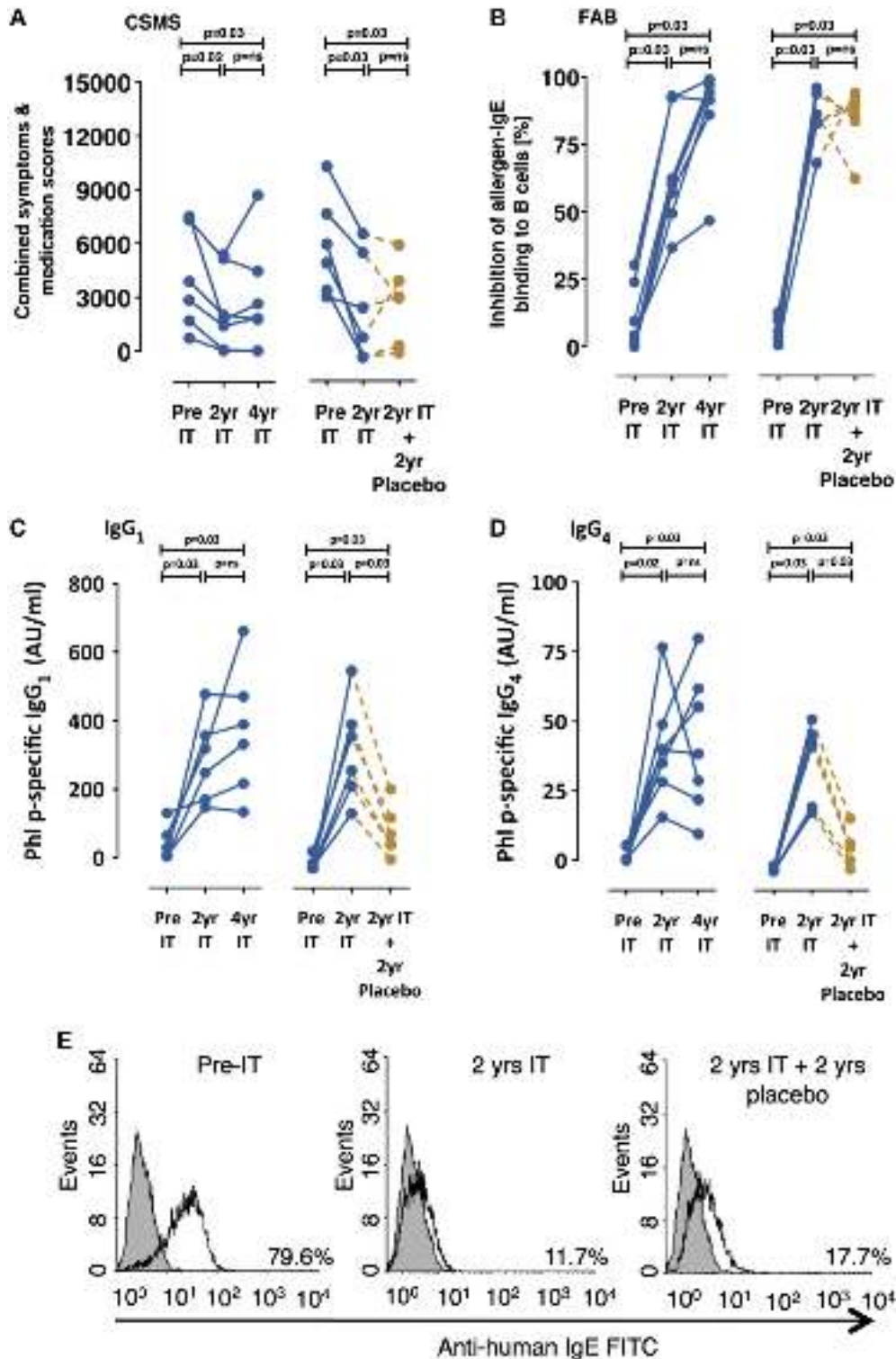
Thus inhibitory bioactivity rather than absolute levels of antibody were associated with clinical tolerance.

The bioassay used to quantify serologic inhibitory activity against IgE is dependent on the formation of allergen-IgE complexes, which are required for efficient IgE binding to low-affinity FcεRII (CD23) receptors on B cells. This validated assay measures the capacity of sera from patients receiving immunotherapy to inhibit formation of these complexes.<sup>16,18,19</sup> We have measured the subsequent inhibition of IgE binding to B cells directly using flow cytometry or indirectly using confocal microscopy to visualize the disaggregation of either surface IgE or CD23. Although immortalized EBV-transformed B cells were routinely used as a replenishable source of live FcεRII-expressing cells, we were also able to demonstrate this mechanism using freshly purified CD19<sup>+</sup> B cells from an allergic subject. This supports our view that this assay is based on a real physiologic IgE-mediated mechanism.

We previously demonstrated that 3 to 4 years of grass pollen immunotherapy is sufficient to induce long-term remission of disease.<sup>8</sup> Our results presented here extend these findings to show that a 2-year course of immunotherapy is also effective. In contrast, a retrospective study of children who were sensitive to house dust mites showed that short-term (12-month) immunotherapy was associated with a greater rate of relapse than was treatment for more than 3 years.<sup>20</sup> We have also previously shown that long-term clinical tolerance after grass pollen immunotherapy is associated with continuing suppression of cutaneous, allergen-induced late-phase responses together with inhibition of local expression of mRNA for T<sub>H</sub>2 cytokines.<sup>8</sup> However, immunologic mechanisms that could account for this long-term

hyporesponsiveness to grass pollen after discontinuation have not previously been described.

Immunotherapy with inhalant allergens is associated with production of allergen-specific IgG1, IgG4, and IgA2 antibodies.<sup>9,21-23</sup> Of these isotypes, allergen-specific IgG4 antibodies show the biggest proportionate increase, increasing within 3 months of starting injections,<sup>9</sup> probably under the influence of newly induced IL-10-producing regulatory T cells.<sup>12,13</sup> Allergen-specific IgG4 antibodies produced in response to pollen immunotherapy directly inhibit allergen- and IgE-dependent cellular responses *in vitro* mediated through low-affinity and high-affinity IgE receptors (FcεRII and FcεRI, respectively), such as facilitated antigen presentation and basophil histamine release.<sup>9,11,17,24-27</sup> These “blocking antibodies” have the potential to account for the clinical efficacy of immunotherapy because the specific neutralization of IgE with omalizumab was shown to reduce seasonal allergic rhinitis symptoms during the pollen season.<sup>18</sup> However, the requirement for blocking antibodies in immunotherapy has been disputed because of weak or absent correlations between serum titers and measures of clinical efficacy.<sup>28-30</sup> Our results during the discontinuation phase also did not show such a correlation because grass pollen-specific IgG1 and IgG4 titers induced after immunotherapy decreased to near-pretreatment levels after 2 years of placebo injections and clinical remission was maintained intact. These findings are robust because they could be completely reproduced in a separate cohort of patients receiving grass pollen immunotherapy who underwent 3 years of placebo-controlled discontinuation, as previously reported (see Fig E3 in this article’s Online Repository at [www.jacionline.org](http://www.jacionline.org)).<sup>8</sup> However, the complete maintenance of serologic



**FIG 3.** Long-term clinical improvement after discontinuation of immunotherapy is associated with persistent IgG-associated blocking activity but not total allergen-specific IgG4 antibodies. **A**, Symptom and medication scores. **B**, Serum inhibitory activity for allergen-IgE binding to B cells. **C** and **D**, *P pratense*-specific IgG1 (Fig 3, C) and IgG4 (Fig 3, D) antibodies measured by means of ELISA. **E**, Representative example of IgE-FAB in the presence of preimmunotherapy and postimmunotherapy serum. CSMS, Combined Symptoms and Rescue Medication Scores; FITC, fluorescein isothiocyanate; IT, immunotherapy.

TABLE II. Total IgE and specific IgE levels

	Immunotherapy-maintained group, mean $\pm$ SE (n = 7)		
	Before immunotherapy	2-y Immunotherapy	4-y Maintained immunotherapy
Phl p 1 and Phl p 5b-specific IgE (kU/L)	32.1 (7.9)	37.5 (11.6)	11.9 (7.4)*
Total IgE (kU/L)	166.7 (62.7)	256.1 (128.1)	226.1 (171.2)
	Immunotherapy-withdrawal group, mean $\pm$ SE (n = 6)		
	Before immunotherapy	2-y Immunotherapy	4-y Withdrawn immunotherapy
Phl p 1 and Phl p 5b-specific IgE (kU/L)	49.8 (21.0)	56.3 (13.2)	45.6 (18.5)
Total IgE (kU/L)	409.3 (166.6)	484.5 (144.5)	993.8 (676.2)

Total and specific IgE antibodies are shown.

\* $P < .03$  compared with preimmunotherapy values (Wilcoxon matched-pairs signed-rank test).

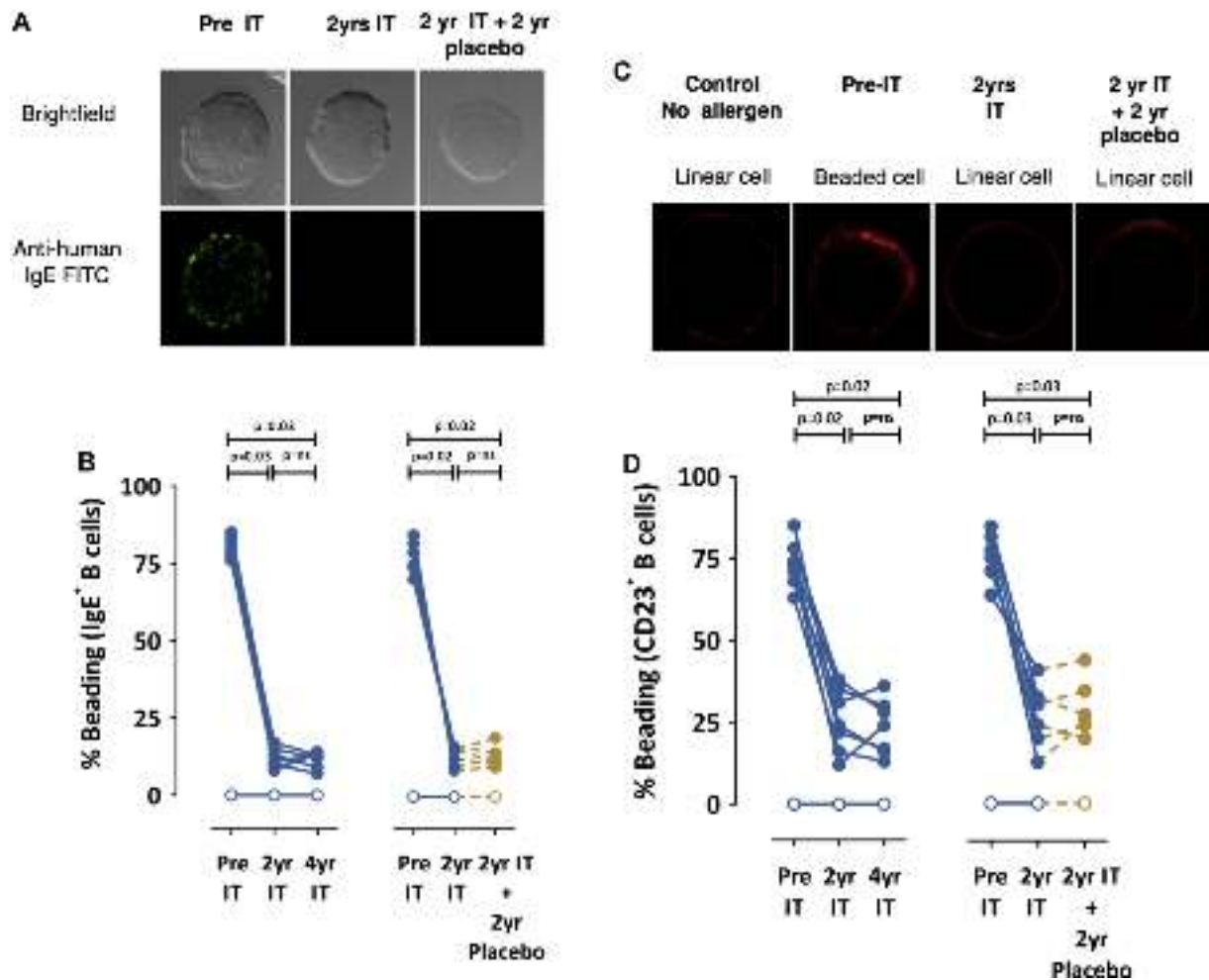
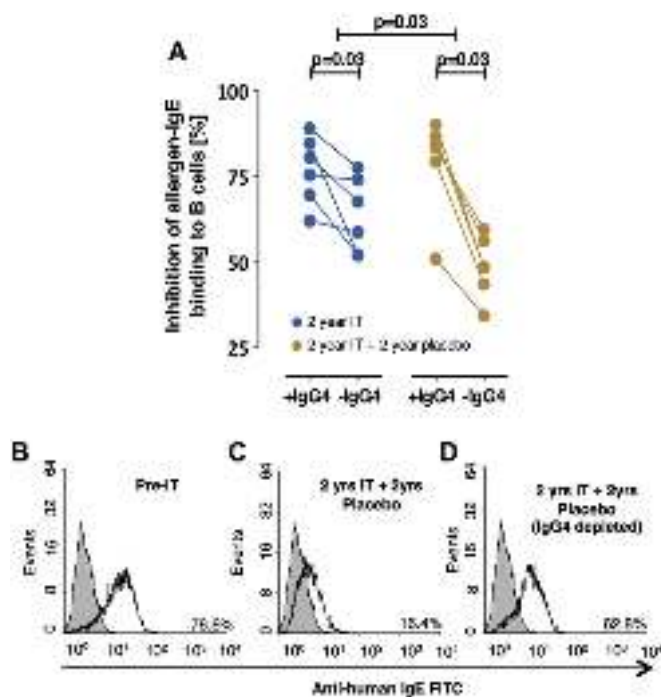


FIG 4. Inhibitory *P pratense*-specific antibodies disrupt IgE binding and CD23 distribution on B cells. **A**, IgE immunostaining. **B**, Quantification of beading pattern (IgE<sup>+</sup> B cells). **C**, CD23 receptor immunostaining. **D**, Quantification of beading pattern (CD23<sup>+</sup> B cells). Controls (without *P pratense*) are represented by open circles. Allergen alone had no effect on B cells (data not shown). FITC, Fluorescein isothiocyanate; IT, immunotherapy.

inhibitory bioactivity after immunotherapy discontinuation suggests a significant dissociation between absolute antibody titers and function.

To explore this further, we depleted IgG4 from serum and identified a reduction in inhibitory activity. Whereas IgG4 did not account entirely for the serum inhibitory activity, we showed that

despite decreasing immunoreactive IgG4 levels, the inhibitory effect persisted for at least 2 years after stopping treatment. We chose to focus on IgG4 because this isotype is induced by IL-10 and we have previously shown that IL-10 levels are increased in the nasal mucosa<sup>17</sup> and in allergen-stimulated peripheral T cells<sup>9</sup> during grass pollen immunotherapy. In contrast to other IgG



**FIG 5.** Inhibition of allergen-IgE complex binding is IgG4 dependent. **A**, IgG4-containing sera versus IgG4-depleted sera. **B** and **C**, Representative examples of allergen-IgE complex binding in the presence of preimmunotherapy and postimmunotherapy serum compared with postimmunotherapy serum depleted of IgG4 antibodies (**D**). FITC, Fluorescein isothiocyanate.

subclasses, IgG4 has distinctive structural and functional characteristics that make it a noninflammatory isotype. For example, IgG4 is able to exchange heavy chains, which results in qualitative “functional” monomeric or bispecific antibodies resulting from exchange of Fab arms.<sup>31</sup> IgG4 has low affinity for Fcγ receptors, does not form immune complexes, is unable to fix complement, and inhibits IgG1-mediated complement activation.<sup>31-33</sup> IgG4 accounted for a proportion (38%) of the serum inhibitory activity in postwithdrawal sera, such that it is likely that other non-IgE antibodies, particularly other IgG subclass antibodies, might contribute to the observed inhibitory effects. Limited availability of serum samples meant that we were only able to perform depletion experiments for 1 IgG isotype. Further studies on withdrawal sera involving depletion of other antibody classes from sera obtained from patients undergoing withdrawal from immunotherapy are needed to address this issue.

In the immunotherapy-maintained group the level of allergen-specific IgE was significantly reduced at 4 years. These results confirm previous findings in relation to ragweed immunotherapy.<sup>34</sup> In the immunotherapy-discontinued group the levels of allergen-specific IgE remained persistently increased, and these participants also remained clinically tolerant. Whereas it is possible that the reduction in FAB-IgE binding at 4 years in the immunotherapy-maintained group could be due in part to a reduction in allergen-specific IgE levels, this is clearly not the case for the immunotherapy-discontinued group in whom IgE levels were maintained, thereby highlighting the importance of blocking factors rather than a reduction in IgE levels alone.

On the basis of these findings we hypothesize that a minority of the IgG antibodies induced by immunotherapy account for the

majority of inhibitory activity against IgE, a process that is revealed during discontinuation of immunotherapy. Because overall immunoreactive IgG levels decrease, the inhibition must relate to altered functional properties of IgG, presumably related to the avidity of IgG-allergen binding. This increased avidity of IgG could be due to either increased affinity to individual IgE epitopes or to an increased number of IgE epitopes that are recognized (ie, increased polyclonality of IgG responses after high allergen exposure during immunotherapy).<sup>35</sup> There are very limited data on measurements of affinity of IgG antibodies after immunotherapy. In one study<sup>36</sup> there were no shifts in overall IgG affinity with whole serum, whole fractionated IgG, or both, although this study was unable to focus on individual monoclonal IgG antibody affinity. Such studies would require cloning of individual IgG-producing B cells from patients before and after immunotherapy.<sup>37,38</sup>

In conclusion, the persistence of potent serum inhibitory activity against IgE, possibly representing high-affinity grass pollen-specific IgG antibodies, suggests a mechanistic explanation for the long-term efficacy of grass pollen immunotherapy. Functional assays, such as those described here, might have value in the design of biomarkers for monitoring and predicting patient status during immunotherapy and after discontinuation of immunotherapy.

We thank Mrs Julia Barkans, Mrs Lavina Saggarr, and Dr Martin Spittler (Facility for Imaging by Light Microscopy [FILM]) for expert technical assistance. We also thank Drs Rob Aalberse and Steven Stapel for providing technical advice on IgG4 depletion experiments.

#### Key message

- Inhibitory IgG4 antibodies persist for up to 2 years after discontinuation of allergen immunotherapy and correlate with clinical outcomes.

#### REFERENCES

- Bauchau V, Durham SR. Prevalence and rate of diagnosis of allergic rhinitis in Europe. *Eur Respir J* 2004;24:758-64.
- Walker S, Khan-Wasti S, Fletcher M, Cullinan P, Harris J, Sheikh A. Seasonal allergic rhinitis is associated with a detrimental effect on examination performance in United Kingdom teenagers: case-control study. *J Allergy Clin Immunol* 2007; 120:381-7.
- Bousquet J, Khaltaev N, Cruz AA, Denburg J, Fokkens WJ, Togias A, et al. Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA2LEN and AllerGen). *Allergy* 2008;63 (suppl 86):8-160.
- White P, Smith H, Baker N, Davis W, Frew A. Symptom control in patients with hay fever in UK general practice: how well are we doing and is there a need for allergen immunotherapy? *Clin Exp Allergy* 1998;28:266-70.
- Wilson AM, Sims EJ, Orr LC, Robb F, Lipworth BJ. An evaluation of short-term corticosteroid response in perennial allergic rhinitis using histamine and adenosine monophosphate nasal challenge. *Br J Clin Pharmacol* 2003;55:354-9.
- Calderon MA, Alves B, Jacobson M, Hurwitz B, Sheikh A, Durham S. Allergen injection immunotherapy for seasonal allergic rhinitis. *Cochrane Database Syst Rev* 2007;(1): CD001936.
- Passalacqua G, Durham SR. Global Allergy and Asthma European Network. Allergic Rhinitis and its impact on Asthma update: allergen immunotherapy. *J Allergy Clin Immunol* 2007;119:881-91.
- Durham SR, Walker SM, Varga EM, Jacobson MR, O'Brien F, Noble W, et al. Long-term clinical efficacy of grass-pollen immunotherapy. *N Engl J Med* 1999; 341:468-75.
- Francis JN, James LK, Paraskevopoulos G, Wong C, Calderon MA, Durham SR, et al. Grass pollen immunotherapy: IL-10 induction and suppression of late

- responses precedes IgG4 inhibitory antibody activity. *J Allergy Clin Immunol* 2008;121:1120-5, e2.
10. Garcia BE, Sanz ML, Gato JJ, Fernandez J, Oehling A. IgG4 blocking effect on the release of antigen-specific histamine. *J Invest Allergol Clin Immunol* 1993;3:26-33.
  11. van Neerven RJ, Wikborg T, Lund G, Jacobsen B, Brinch-Nielsen A, Arved J, et al. Blocking antibodies induced by specific allergy vaccination prevent the activation of CD4+ T cells by inhibiting serum-IgE-facilitated allergen presentation. *J Immunol* 1999;163:2944-52.
  12. Satoguina JS, Adjobimey T, Arndts K, Hoch J, Oldenburg J, Layland JE, et al. Tr1 and naturally occurring regulatory T cells induce IgG4 in B cells through GITR/GITR-L interaction, IL-10 and TGF-beta. *Eur J Immunol* 2008;38:3101-13.
  13. Meiler F, Klunker S, Zimmermann M, Akdis CA, Akdis M. Distinct regulation of IgE, IgG4 and IgA by T regulatory cells and toll-like receptors. *Allergy* 2008;63:1455-63.
  14. Radulovic S, Jacobson MR, Durham SR, Nouri-Aria KT. Grass pollen immunotherapy induces Foxp3-expressing CD4+ CD25+ cells in the nasal mucosa. *J Allergy Clin Immunol* 2008;121:1467-72.
  15. Walker SM, Pajno GB, Lima MT, Wilson DR, Durham SR. Grass pollen immunotherapy for seasonal rhinitis and asthma: a randomized, controlled trial. *J Allergy Clin Immunol* 2001;107:87-93.
  16. Shamji MH, Wilcock LK, Wachholz PA, Dearman RJ, Kimber I, Wurtzen PA, et al. The IgE-facilitated allergen binding (FAB) assay: validation of a novel flow-cytometric based method for the detection of inhibitory antibody responses. *J Immunol Methods* 2006;317:71-9.
  17. Nouri-Aria KT, Wachholz PA, Francis JN, Jacobson MR, Walker SM, Wilcock LK, et al. Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. *J Immunol* 2004;172:3252-9.
  18. Klunker S, Saggat LR, Seyfert-Margolis V, Asare AL, Casale TB, Durham SR, et al. Combination treatment with omalizumab and rush immunotherapy for ragweed-induced allergic rhinitis: Inhibition of IgE-facilitated allergen binding. *J Allergy Clin Immunol* 2007;120:688-95.
  19. Wachholz PA, Soni NK, Till SJ, Durham SR. Inhibition of allergen-IgE binding to B cells by IgG antibodies after grass pollen immunotherapy. *J Allergy Clin Immunol* 2003;112:915-22.
  20. Des Roches A, Paradis L, Knani J, Hejjoui A, Dhivert H, Chanez P, et al. Immunotherapy with a standardized *Dermatophagoides pteronyssinus* extract. V. Duration of the efficacy of immunotherapy after its cessation. *Allergy* 1996;51:430-3.
  21. McHugh SM, Lavelle B, Kemeny DM, Patel S, Ewan PW. A placebo-controlled trial of immunotherapy with two extracts of *Dermatophagoides pteronyssinus* in allergic rhinitis, comparing clinical outcome with changes in antigen-specific IgE, IgG, and IgG subclasses. *J Allergy Clin Immunol* 1990;86:521-31.
  22. Gehlar K, Schlaak M, Becker W, Bufe A. Monitoring allergen immunotherapy of pollen-allergic patients: The ration of allergen-specific IgG4 to IgG1 correlates with clinical outcome. *Clin Exp Allergy* 1999;29:497-506.
  23. Rossi RE, Monasterolo G, Coco G, Silvestro L, Operti D. Evaluation of serum IgG4 antibodies specific to grass pollen allergen components in the follow up of allergic patients undergoing subcutaneous and sublingual immunotherapy. *Vaccine* 2007;15:957-64.
  24. Lambin P, Bouzoumou A, Murrieta M, Debbia M, Rouger P, Leynadier F, et al. Purification of human IgG4 subclass with allergen-specific blocking activity. *J Immunol Methods* 1993;165:99-111.
  25. Strait RT, Morris SC, Finkelman FD. IgG-blocking antibodies inhibit IgE-mediated anaphylaxis in vivo through both antigen interception and Fc gamma RIIb cross-linking. *J Clin Invest* 2006;116:833-41.
  26. Wurtzen PA, Lund G, Lund K, Arvidsson M, Rak S, Ipsen H. A double-blind placebo-controlled birch allergy vaccination study II: correlation between inhibition of IgE binding, histamine release and facilitated allergen presentation. *Clin Exp Allergy* 2008;38:1290-301.
  27. Cady CT, Powell MS, Harbeck RJ, Giclas PC, Murphy JR, Katial RK, et al. IgG antibodies produced during subcutaneous allergen immunotherapy mediate inhibition of basophil activation via a mechanism involving both Fc gamma RIIA and Fc gamma RIIb. *Immunol Lett* 2010;130:57-65.
  28. Muller U, Akdis CA, Fricker M, Akdis M, Blesken T, Bettens F, et al. Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom. *J Allergy Clin Immunol* 1998;101:747-54.
  29. Ewan PW, Deighton J, Wilson AB, Lachmann PJ. Venom-specific IgG antibodies in bee and wasp allergy: lack of correlation with protection from stings. *Clin Exp Allergy* 1993;23:647-60.
  30. Djurup R, Sondergaard I, Magnusson CG, Weeke B. A three-layer immunoradiometric assay for determination of IgG subclass antibodies in human sera ("IgG subclass RAST"). Validation of the subclass specificity, and establishment of equipotency. *Allergy* 1984;39:51-63.
  31. van der Neut Kofschoten M, Schuurman J, Losen M, Bleeker WK, Martínez-Martínez P, Vermeulen E, et al. Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange. *Science* 2007;317:1554-7.
  32. Aalberse RC, Schuurman J. IgG4 breaking the rules. *Immunology* 2002;105:9-19.
  33. Aalberse RC, van der Gaag R, van Leeuwen J. Serologic aspects of IgG4 antibodies. I. Prolonged immunization results in an IgG4-restricted response. *J Immunol* 1983;130:722-6.
  34. Gleich GJ, Zimmermann EM, Henderson LL, Yunginger JW. Effect of immunotherapy on immunoglobulin E and immunoglobulin G antibodies to ragweed antigens: a six-year prospective study. *J Allergy Clin Immunol* 1982;70:261-71.
  35. Michils A, Baldassarre S, Ledent C, Mairesse M, Gossart B, Duchateau J. Early effect of ultrarush venom immunotherapy on the IgG antibody response. *Allergy* 2000;55:455-62.
  36. Svenson M, Jacobi HH, Bødtger U, Poulsen LK, Rieneck K, Bendtzen K. Vaccination for birch pollen allergy. Induction of affinity-matured or blocking IgG antibodies does not account for the reduced binding of IgE to Bet v 1. *Molecular Immunology* 2003;39:603-12.
  37. Tiller T, Meffre E, Yurasov S, Tsuiji M, Nussenzweig MC, Wardemann H. Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *J Immunol Methods* 2008;329:112-24.
  38. Smith K, Garman L, Wrammert J, Zheng NY, Capra JD, Ahmed R, et al. Rapid generation of fully human monoclonal antibodies specific to a vaccinating antigen. *Nat Protoc* 2009;4:372-84.

## METHODS

### Pollen counts

Numbers of grass pollen grains per cubic meter of air were recorded daily throughout the pollen season (May-August) by using a Burkard 7-day volumetric spore trap on a London rooftop (18 m high), and mean weekly counts were calculated (Fig 1, A).

### Antibodies

The following antibodies were purchased from DakoCytomation Ltd (Ely, United Kingdom): polyclonal rabbit fluorescein isothiocyanate-conjugated anti-human IgE (1.9 g/L), phycoerythrin-conjugated anti-human CD23 mAb (100 mg/L), and an isotype control (murine IgG1-phycoerythrin, 100 mg/L).

### Atopic serum

Serum containing high concentration of *P pratense*-specific IgE (>100 IU/mL) obtained from a single grass pollen-sensitized donor was purchased from PlasmaLab International (Everett, Wash). This indicator serum was used in all experiments measuring allergen-IgE binding to B cells to provide interassay reproducibility when assessing inhibitory antibody responses.

### CD23-enriched EBV-transformed B cells

CD23-enriched EBV-transformed B cells (CD14<sup>-</sup>, CD19<sup>+</sup>, CD20<sup>+</sup>, CD23<sup>+</sup>, CD25<sup>-</sup>, CD3<sup>-</sup>, CD40<sup>+</sup>, CD80<sup>+</sup>, CD86<sup>+</sup>, and HLA-DR<sup>+</sup> cells) were a kind gift from ALK-Abelló. The cell line was maintained in vented-cap, canted-neck cell-culture flasks (Falcon/VWR, Poole, United Kingdom) at 37°C, 5% CO<sub>2</sub>, and 95% relative humidity. Cells were subcultured 2 to 3 times a week to maintain a cell density of 0.5 to 1.5 × 10<sup>6</sup> cells/mL in RPMI-1640 supplemented with 1% (vol/vol) L-glutamine, 10% heat-inactivated FCS, and 1% (vol/vol) penicillin/streptomycin mixture (Invitrogen, Paisley, United Kingdom). The viability of healthy, growing, CD23-enriched EBV-transformed B cells were assessed by using trypan blue dye (Sigma-Aldrich). Cells were counted on a Neubauer hemocytometer with a light microscope at ×40 magnification. Nonviable cells were considered to be those that took up the blue dye, and viable cells remained colorless.

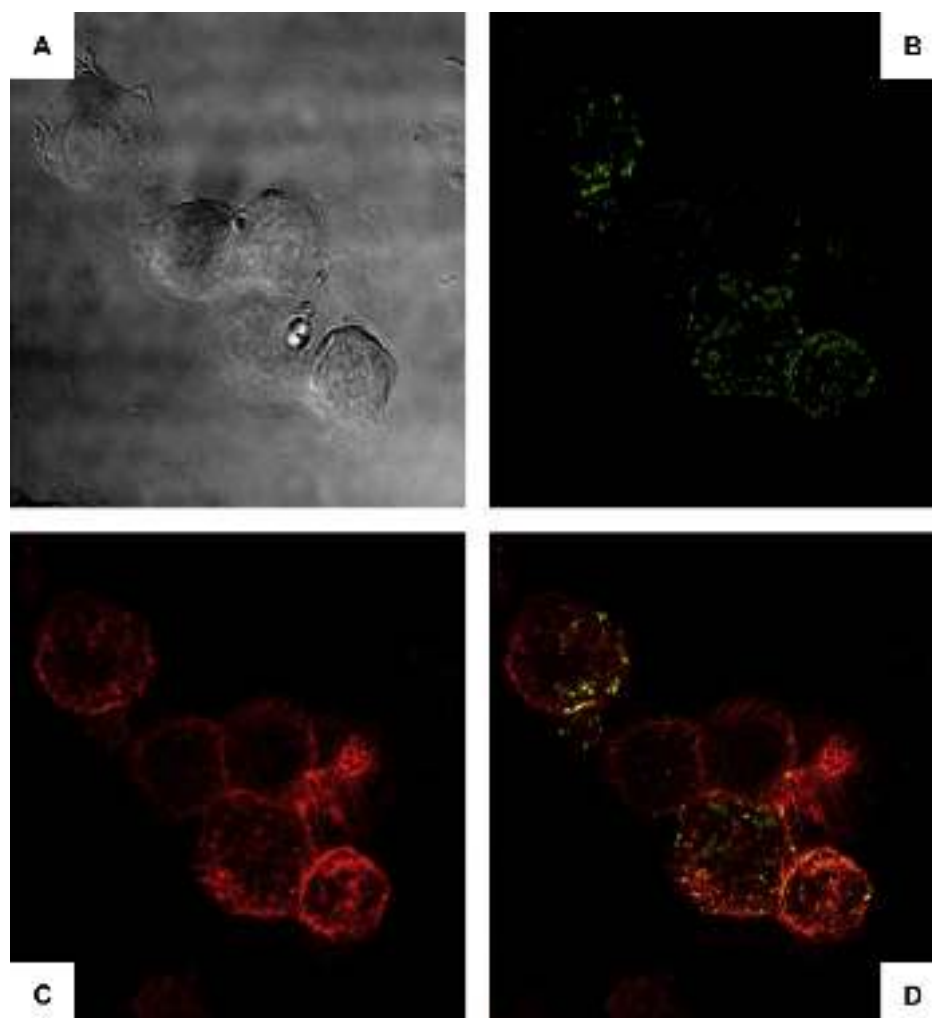
The typical viability of the CD23-enriched EBV-transformed B cells was between 98% and 100%.

### Visualization of allergen-IgE complexes and CD23-IgE-allergen receptor complexes on surfaces of B cells

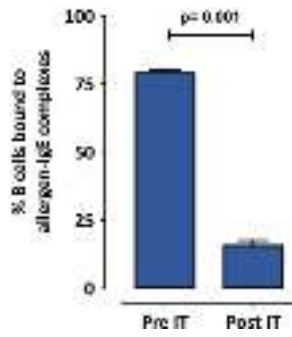
Twenty microliters of stock indicator serum containing a high concentration of *P pratense*-specific IgE (>100 IU/mL) was preincubated with 5 μL of 1 μg/mL allergen at 37°C for 1 hour to allow formation of allergen-IgE complexes. Twenty microliters of indicator serum and 20 μL of test serum or RPMI as a control were mixed to test for inhibition of FAB. During this step, CD23-enriched EBV-transformed B cells were washed 3 times by means of centrifugation in RPMI-1640 at 423g for 7 minutes at 4°C. Cells were then resuspended in FAB buffer (138.60 mmol/L NaCl, 1.12 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 8.16 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, and 0.1% BSA dissolved in 1 L of distilled H<sub>2</sub>O, with pH adjusted to 7.2) at 2 × 10<sup>7</sup> cells/mL.

EBV-transformed B cells (1 × 10<sup>5</sup>) were added to the IgE-allergen complexes and incubated for 1 hour at 4°C on ice. Cells were then washed twice in FAB buffer by means of centrifugation at 423g for 5 minutes at 4°C to remove any unbound allergen-IgE complexes. Cells were resuspended in FAB buffer and stained with fluorescein isothiocyanate-labeled anti-human IgE antibody or phycoerythrin-labeled anti-human CD23 for 45 minutes at 4°C on ice. The cells were then washed and fixed in 4% paraformaldehyde for 5 minutes.

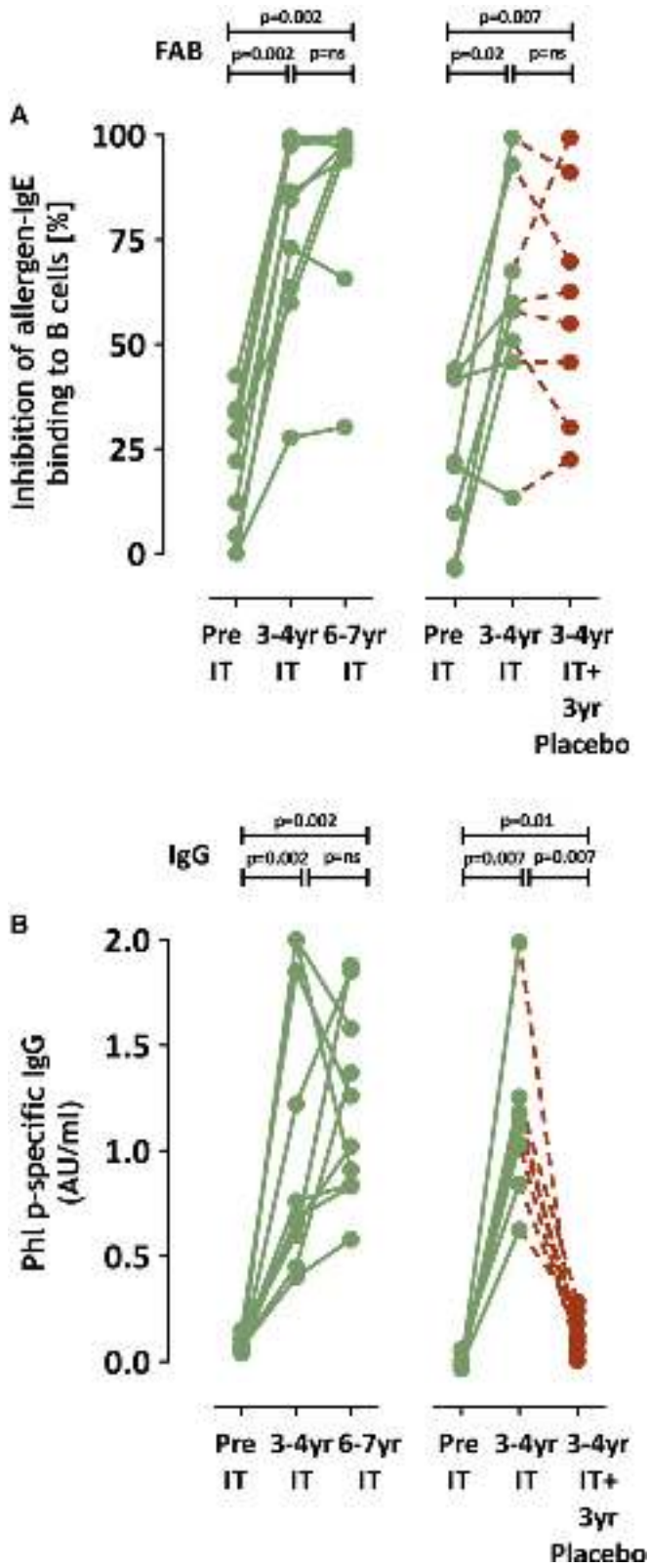
Cells then underwent cytospin preparation in polylysine-coated slides (Superfrost Plus; Fisher Scientific, Cambridge, United Kingdom) at 400 rpm for 2 minutes. Slides were mounted in fluorescent medium. Visualization of allergen-IgE complexes and CD23 receptor distribution was achieved by using a ×63 DIC lens on a Zeiss LSM-510 inverted confocal laser scanning microscope (Carl Zeiss). All images were acquired by using LSM 4.0 analysis software, and a standardized digital setting was used when comparing staining between samples. All samples were blinded before analysis. The percentage of allergen-IgE complex-positive B cells was visualized and qualified as a beaded pattern. Moreover, the percentage of CD23<sup>+</sup> B cells with beading and the linear or circumferential pattern were quantified in samples.



**FIG E1.** Visualization of CD23 receptor coaggregation and allergen-IgE complexes binding to B cells. Serum from a subject with grass allergy (*P pratense*-specific IgE >100 IU/L) was incubated with 1  $\mu$ g/mL *P pratense* extract in the presence of preimmunotherapy serum. CD23-enriched EBV-transformed B cells were incubated with the complexes. Cells were immunostained with fluorescein isothiocyanate-labeled rabbit polyclonal anti-human IgE antibody and phycoerythrin-labeled antihuman CD23 antibody. **A**, A bright field image of the cells is shown. **B**, Allergen-IgE complexes were visualized as IgE<sup>+</sup> staining. **C**, Clustering of CD23 receptors were visualized as a beading pattern. **D**, Colocalization of CD23-IgE-allergen complexes with dual staining of CD23 receptor and allergen-IgE complexes.



**FIG E2.** CD23-dependent allergen binding with freshly purified B cells. IgE-FAB to B cells isolated from PBMCs was measured by using sera from preimmunotherapy ( $n = 5$ ) and postimmunotherapy ( $n = 5$ ) subjects. Addition of postimmunotherapy serum resulted in a significant decrease of allergen-IgE complexes binding to B cells isolated from PBMCs.  $P$  values represent statistical differences within groups, as determined by using the Wilcoxon matched-pairs test. *IT*, Immunotherapy.



**FIG E3.** Persistence of functional *P pratense*-specific IgG-dependent inhibitory antibody activity after discontinuation of grass pollen immunotherapy. **A**, Serum-inhibitory activity for allergen-IgE binding to B cells. **B**, *P pratense*-specific IgG antibodies measured by using ELISA. These data were obtained from a second cohort of patients who underwent discontinuation of grass pollen immunotherapy. Originally described in Durham et al.<sup>8</sup> IT, Immunotherapy.

**TABLE E1.** Total and *P pratense*-specific IgG4 levels before and after IgG4 depletion

	Serum before depletion, median (IQR)	Serum after depletion, median (IQR)	Two-tailed <i>P</i> value
Postimmunotherapy serum (4-year IT)			
Antigen-specific IgG4 (AU/mL)	1.61 (0.50-2.12)	0.07 (0.07-0.07)	.03
Total IgG4 (g/L)	0.37 (0.20-0.50)	0 (0.00-0.00)	.03
Postwithdrawal serum (2-y IT + 2-y placebo)			
Antigen-specific IgG4 (AU/mL)	0.24 (0.09-0.36)	0.07 (0.07-0.08)	.03
Total IgG4 (g/L)	0.23 (0.1-0.4)	0 (0.00-0.00)	.03

Serum samples taken from the placebo-controlled withdrawal group (after immunotherapy and after withdrawal) were passed over an anti-IgG4 Sepharose column. Depletion of allergen-specific IgG4 antibodies was confirmed by means of ELISA and total IgG4 by means of nephelometry. Statistical significance was assessed by using the Wilcoxon matched signed-rank test.

*AU*, Arbitrary units.