Grass pollen immunotherapy: IL-10 induction and suppression of late responses precedes IgG4 inhibitory antibody activity

James N. Francis, PhD,* Louisa K. James, PhD,* Giannis Paraskevopoulos, MD, Cheukyee Wong, BSc, Moises A. Calderon, MD, PhD, Stephen R. Durham, MD, and Stephen J. Till, MD, PhD *London, United Kingdom*

Background: Grass pollen immunotherapy is an effective treatment for seasonal allergic rhinitis that provides the opportunity to study the induction and maintenance of allergen-specific immune tolerance.

Objectives: We investigated the relationship between clinical responsiveness, regulatory cytokine production, and antibody responses to allergen during 1 year of immunotherapy.

Methods: Eighteen subjects with severe seasonal allergic rhinitis were randomized double-blind to receive active or placebo injections of an alum-adsorbed grass pollen vaccine (Alutard SQ). Subjects underwent repeated testing of early- and late-phase skin responses to intradermal allergen, and cellular responses to grass pollen allergen were tested. Sera were tested for allergen-specific IgG4, IgA, and inhibitory activity in biologic assays of IgE responses.

Results: Grass pollen immunotherapy was effective in reducing overall symptom scores ($P < .05$) and conjunctival reactivity ($P < .05$). In the active group significant IL-10 production occurred early at low allergen doses and at a similar time as inhibition of late skin responses at 2 to 4 weeks. Serum allergen-specific IgG4, IgA, and inhibitory antibody activity for basophil histamine release and IgE-facilitated allergen binding to B cells occurred later, at 6 to 12 weeks, at higher allergen doses and preceded inhibition of early skin responses.

Conclusion: IL-10 responses occur early but at immunotherapy doses that are not clinically effective. Later induction of inhibitory antibodies, including IgG4 and IgA, might be required for efficacy through modulation of IgE-mediated events. (J Allergy Clin Immunol 2008;121:1120-5.)

Key words: *Immunotherapy, IL-10, late-phase response, IgG4, IgA, early-phase response*

Allergic rhinitis affects a quarter of the population in countries with a Western lifestyle[1] and represents a major cause of morbidity, affecting social functioning, sleep quality, and performance at school or work.[2] Although pharmacotherapy can be effective,[3] there remains a substantial proportion of subjects whose symptoms are inadequately controlled[4] and in whom allergen-specific immunotherapy (desensitization) is currently recommended.[5-7] We previously demonstrated that 3 to 4 years of immunotherapy for severe seasonal allergic rhinitis is highly effective in inducing remission for at least 3 years after discontinuation.[8] Immunotherapy is therefore able to modify the course of allergic disease and is the only antigen-specific immunomodulatory treatment in routine use.

Immunotherapy induces both cellular and humoral regulatory mechanisms.[9] Cellular mechanisms involve IL-10, a cytokine expressed after immunotherapy by nasal mucosal[10] and peripheral blood $CD4^+CD25^+$ cells, possibly regulatory T cells.[11,12] IL-10 has numerous important anti-inflammatory properties, including inhibition of T-cell cytokine production and activation of mast cells and eosinophils.[13,14] Humoral mechanisms involve allergen-specific antibodies, especially of the IgG4 isotype, that inhibit IgE-mediated histamine release[15] and antigen presentation[16] by means of direct allergen competition and through inhibitory FcγRIIb IgG receptors.[17]

The evolution of the immunologic response to immunotherapy in relation to clinical markers of allergen desensitization has never been accurately defined. We hypothesized that IL-10 production is an early event inducing allergen-specific inhibitory IgG4 responses that contribute to inhibition of early and late responses and that are essential for clinical efficacy. To test these hypotheses, subjects with severe seasonal allergic rhinitis were randomized to receive 12 months of active treatment with grass pollen immunotherapy or placebo injections.

Cutaneous early- and late-phase responses, cytokine responses in PBMC cultures, immunoreactive antibodies, and biologic inhibitory antibody function were assayed at regular intervals.

METHODS

Patient selection

Patients with seasonal allergic rhinitis were selected on the basis of a history of moderate/severe seasonal allergic rhinitis and poor symptom control

From the Allergy and Clinical Immunology Section, National Heart and Lung Institute, Imperial College London.

*These authors contributed equally to this work.

Supported by grants from the Immune Tolerance Network, National Institutes of Health; Asthma UK; and GlaxoSmithKline in partnership with Imperial College Trust (academic Drug Discovery Initiative) and ALK-Abelló, Hørsholm, Denmark. S.J.T. is the recipient of a Clinician Scientist Fellowship supported by the Health Foundation.

Disclosure of potential conflict of interest: M. A. Calderon has consulting arrangements with ALK-Abelló, MSD, and Schering-Plough and has received research support from ALK-Abelló. S. R. Durham has consulting arrangements with ALK-Abelló and GlaxoSmithKline, has received research support from ALK-Abelló and GlaxoSmithKline, and has served as a member of the Immune Tolerance Network and the National Institutes of Health. The rest of the authors have declared that they have no conflict of interest.

Received for publication July 31, 2007; revised January 11, 2008; accepted for publication January 21, 2008.

Available online March 28, 2008.

Reprint requests: Stephen J. Till, MD, PhD, Allergy and Clinical Immunology Section, National Heart and Lung Institute, Imperial College London, Sir Alexander Fleming Building, London, SW7 2AZ, United Kingdom. E-mail: stephen.till@imperial.ac.uk.

0091-6749/$34.00

doi:10.1016/j.jaci.2008.01.072

TABLE I. Subject characterization

	Immunotherapy (n = 12)	Placebo (n = 6)
Age (y)	30.5 ± 1.7	37.0 ± 5.8
Sex (male/female)	7/5	2/4
Total IgE (IU/mL)	346 ± 103	124 ± 24
Specific IgE out of season (IU/mL)	48.3 ± 9.1	43.7 ± 12.7
Specific IgE in season (IU/mL)	60.9 ± 10.1	63.6 ± 13.1
Skin prick test (mm)	10.9 ± 0.9	10.3 ± 1.1

Data are expressed as means ± SEs.

in previous years despite pharmacotherapy (see the Methods section in the Online Repository at www.jacionline.org for further details). Participants had a positive skin prick test response (wheal >5 mm) to timothy grass pollen extract (Soluprick SQ; ALK-Abelló, Hørsholm, Denmark). Patients were excluded if they were polysensitized with perennial symptoms or had a clinical history of chronic asthma or other significant medical illness. Patients with mild seasonal asthma were included, provided their symptoms were adequately controlled with inhaled β_2-adrenergic agonist bronchodilators. Studies were performed with the approval of the Royal Brompton and Harefield Hospitals NHS Trust Ethics Committee. All patients provided written informed consent.

Study design

Before the pollen season of 2003, 18 patients (Table I) underwent double-blind randomization (2:1) to receive timothy grass pollen (whole extract) immunotherapy (Alutard SQ, ALK-Abelló; n = 12) or placebo injections (n = 6) for 12 months (see the Methods section of the Online Repository for details of immunotherapy). No significant differences in clinical characteristics were observed between the active and placebo groups. All patients underwent repeated venesection and intradermal allergen challenge for testing of early and late skin responses at biweekly intervals during updosing and at monthly intervals during the maintenance phase. In all cases there was a minimum 1-week interval between peripheral blood collection and the preceding immunotherapy injection. On each occasion, intradermal allergen injections were timed such that late responses could be recorded shortly before an immunotherapy injection was administered. Intradermal testing was performed on the extensor surface of the forearm with an injection of 33.3 SQ-U of allergen (equivalent to 2 ng of the major grass allergen Phl p 5 or 10 BU) in 0.02 mL of diluent with a negative control injection of diluent alone. Early responses were recorded after 15 minutes as the mean diameter of the wheal, and late responses were recorded as the mean diameter of swelling at 24 hours. All measurements were performed by a single clinician under double-blind conditions. On study completion, all patients underwent conjunctival provocation testing (see the Methods section in the Online Repository), and overall assessment scores were obtained. Patients responded to the question "How has your hay fever been this year compared with previous years?" on a numeric scale from −3 (a lot worse) to +3 (a lot better).

Immunologic tests

PBMCs were isolated and cultured for 6 days, as previously described, and cytokine concentrations were measured in culture supernatants by means of ELISA.[11] Serum allergen-specific IgE levels were measured by using RASTs with Sepharose-coupled grass pollen allergen and detection by using radiolabeled anti-IgE. Serum allergen-specific IgG4 and IgA levels were measured by means of ELISA (coating allergen concentration, 5 μg/mL; 1:100 or 1:20 dilution of sera, respectively; detection with biotinylated anti-human IgG4 or IgA mAb; BD PharMingen, San Diego, Calif). For histamine release experiments, whole blood from a grass pollen–sensitive donor was incubated with 1 pg/mL whole *Phleum pratense* extract (ALK-Abelló) in the presence or absence of sera from study patients (see the Methods section of the Online Repository). Histamine concentrations in cell-free supernatants were measured by means of ELISA (IBL, Hamburg, Germany). IgE-facilitated allergen binding to B cells was performed as previously described.[18,19] Briefly, serum from a grass pollen–sensitive donor (containing high concentrations of allergen-specific IgE) was incubated with 3 μg/mL *P pratense* in the presence or absence of an equal volume of sera from study patients for 1 hour at 37°C. EBV-transformed B cells (1×10^5) were added for 1 hour at 4°C and washed, and allergen–IgE binding was detected by using a fluorescein isothiocyanate–labeled polyclonal anti-IgE antibody. Allergen–IgE binding to B cells was analyzed by means of flow cytometry.

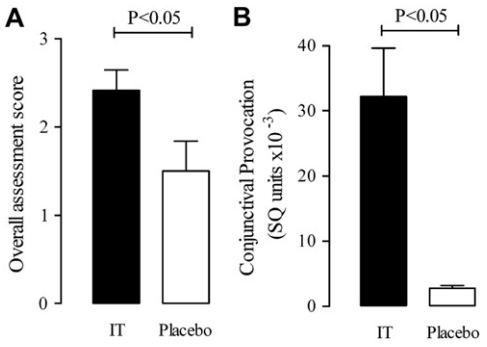

FIG 1. Grass pollen immunotherapy results in reduced symptoms and decreased conjunctival reactivity to allergen. Eighteen patients were randomized to receive active immunotherapy (*IT*; n = 12) or placebo injections (n = 6) for 1 year. **A**, Overall assessment of symptoms was recorded on a numeric scale (−3 to +3) by each patient at the end of the study. **B**, Conjunctival provocation testing was performed at the end of the study.

Statistical analysis

Based on our previous studies,[8] inclusion of 6 participants per group resulted in a greater than 90% chance of detecting a 50% reduction in the late response at 12 months after immunotherapy. An additional 6 subjects were included to receive active immunotherapy (2:1 randomization; 12 participants receiving active therapy and 6 receiving placebo therapy). Data were normally distributed, as determined by using the Kolmogorov-Smirnov test for normality, and are reported as means ± SE. Within-group comparisons with baseline measurements were performed by using repeated-measures 1-way ANOVA, followed by the Bonferroni multiple comparison test. For overall comparison of data from patients receiving immunotherapy and those receiving placebo, area under the curve values were calculated for the total period of treatment after achieving the maintenance dose (ie, during the entire 10-month maintenance phase) and were compared by using the Student *t* test (with the Welch correction when variance of the 2 data groups was unequal, as determined by using an F test). All tests were 2-tailed. *P* values of less than .05 were considered to indicate statistical significance.

RESULTS

Overall clinical and immunologic changes during allergen immunotherapy

Subjects who received grass pollen immunotherapy during 2003 had a significant clinical response to treatment, with reduced symptoms and responsiveness to grass pollen allergen compared with those seen in the placebo group. After 1 year's treatment, patients receiving immunotherapy had significantly better mean (±SE) overall assessment scores (2.42 ± 0.22) compared with patients receiving placebo (1.50 ± 0.34, $P < .05$; Fig 1, *A*). Patients receiving immunotherapy also required significantly greater concentrations of *P pratense* allergen to elicit conjunctival symptoms on provocation testing (32,182 ± 7432 SQ-U/mL) than patients receiving placebo (2760 ± 440 SQ-U/mL, $P < .05$; Fig 1, *B*). Skin early and late responses to intradermal allergen challenge, allergen-induced cytokine production by PBMCs,

TABLE II. Overall analysis of active and placebo treatment

	Active (n = 12)	Placebo (n = 6)	*P* value*
Skin early response (m^2/mo)	1.32 ± 0.80	1.76 ± 0.15	<.05
Skin late response (m^2/mo)	2.26 ± 0.53	14.20 ± 3.39	<.001
IL-10 (μg/mL/mo)	1.33 ± 0.27	0.51 ± 0.10	<.05
IL-5 (μg/mL/mo)	3.22 ± 0.56	1.74 ± 0.66	.13
IFN-γ (μg/mL/mo)	7.14 ± 1.67	6.30 ± 2.80	.78
Specific IgG4 (AU/mL/mo)	86.7 ± 23.7	11.9 ± 3.15	<.05
Specific IgE (AU/mL/mo)	324 ± 921	242 ± 470	.56
Inhibition of IgE-facilitated allergen binding (%/mo)	329 ± 24	162 ± 39	<.01
Inhibition of histamine release (%/mo)	380 ± 34	202 ± 51	<.01

Data are expressed as the mean area under the curve for the maintenance phase of treatment (± SEs).
*Between-group comparisons were performed by using the Student unpaired *t* test with the Welch correction where appropriate.

serum allergen-specific antibodies, inhibition of IgE-facilitated allergen binding to B cells, and inhibition of basophil histamine release during the 10-month maintenance phase of injections are presented in Table II. All parameters were significantly different between patients receiving immunotherapy and patients receiving placebo, with the exception of *P pratense*–specific IgE and allergen-induced IL-5 and IFN-γ production.

Time course of early- and late-phase skin responses

The temporal relationships between early- and late-phase responses to intradermally administered *P pratense* were examined (Fig 2, *A*). Before starting treatment, the mean (±SE) late skin response measured 24 hours after intradermal allergen challenge was 9519 ± 1132 mm^2 (Fig 2, *C*). In the active group, on repeated measurement after only 2 weeks, late responses were significantly reduced (6383 ± 918 mm^2; $P < .001$, a 33% reduction compared with baseline values). At this point in the study, patients receiving immunotherapy had received only a total cumulative dose of 3100 SQ-U (equivalent to approximately 0.6 μg of the major grass allergen Phl p 5) administered as 4 separate injections (see Table E1 in the Online Repository at www.jacionline.org), with the last injection being 1 week before assessment of late responses. Compared with the late response, reductions in early skin responses occurred much later in treatment and were proportionately smaller (Fig 2, *B*). A significant reduction in mean (±SE) early responses was recorded at week 12/April (baseline, 545 ± 65 mm^2; week 12, 398 ± 49 mm^2; $P < .01$, 27% reduction compared with baseline value), with each subject having received a cumulative dose of 398,100 SQ-U of grass pollen allergen. Overall, greater than 90% suppression of the late-phase response was achieved at 12 weeks, whereas maximal suppression of the early-phase response was only 44% and occurred later at 22 weeks. In the placebo group a 53% reduction of the late-phase response occurred by 6 weeks ($P < .05$) compared with baseline measurements, although no further changes were observed for the remainder of the study period (Fig 2, *C*). No significant changes in early-phase responses occurred in the placebo group (Fig 2, *B*).

Serum antibodies

In the active group serum *P pratense*–specific IgG4 levels were significantly increased from baseline measurements by week 12

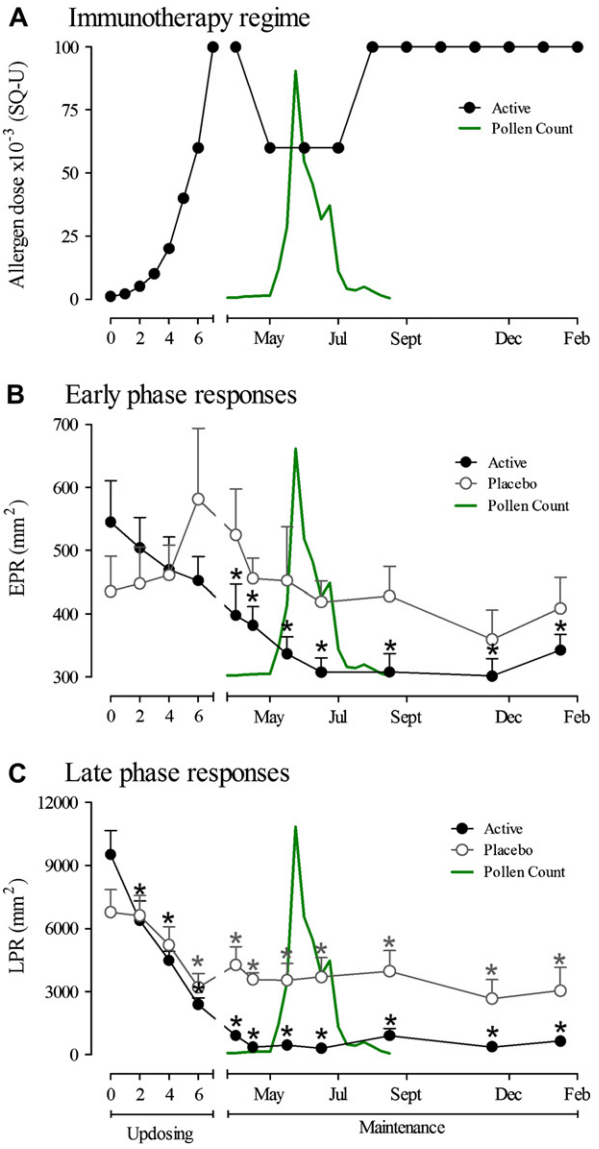

FIG 2. Time-course analysis of clinical measurements during the first year of grass pollen immunotherapy. The dose of grass pollen allergen administered at each immunotherapy visit is represented in **A**. Early (**B**) and late (**C**) skin responses were assessed at 15 minutes and 24 hours after intradermal challenge with grass pollen allergen. Data are normalized to time after initial injection for the first 8 weeks of treatment, and thereafter data are plotted against collection date to assess potential effects of the grass pollen season. Data are expressed as means ± SEs and analyzed by using ANOVA with the Bonferroni correction. *Primary significance from baseline. *EPR*, Early-phase response; *LPR*, late-phase response.

(baseline, 4.52 ± 3.41 AU/mL; week 12, 16.81 ± 4.36 AU/mL; $P < .01$; Fig 3, *A*). Significant increases in IgG4 levels were also observed in the placebo group (baseline, 1.13 ± 0.54 AU/mL; May, 2.37 ± 0.68 AU/mL; $P < .05$) and remained significantly different from baseline measurements until August. Maximal IgG4 levels in the placebo group were approximately 10-fold lower compared with those in the active group. A similar pattern of serum allergen-specific IgA levels was observed in the active group, with significant increases occurring at 12 weeks (baseline, 6.81 ± 1.89 AU/mL; week 12, 42.82 ± 15.86 AU/mL; $P < .05$), although no changes in IgA levels were detected in the placebo

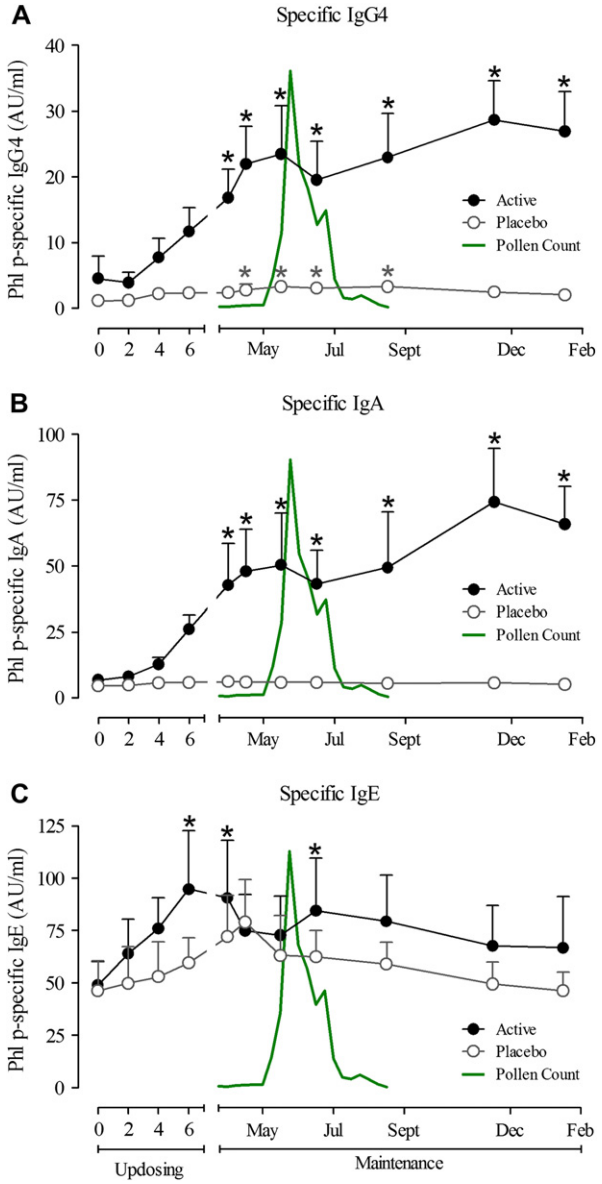

FIG 3. Time-course analysis of serum antibody measurements during the first year of grass pollen immunotherapy. Allergen-specific IgG4 (**A**) and IgA (**B**) levels were measured in serum by means of ELISA, and IgE was measured by using RAST (**C**). Data are normalized to time and analyzed as previously. *Primary significance from baseline.

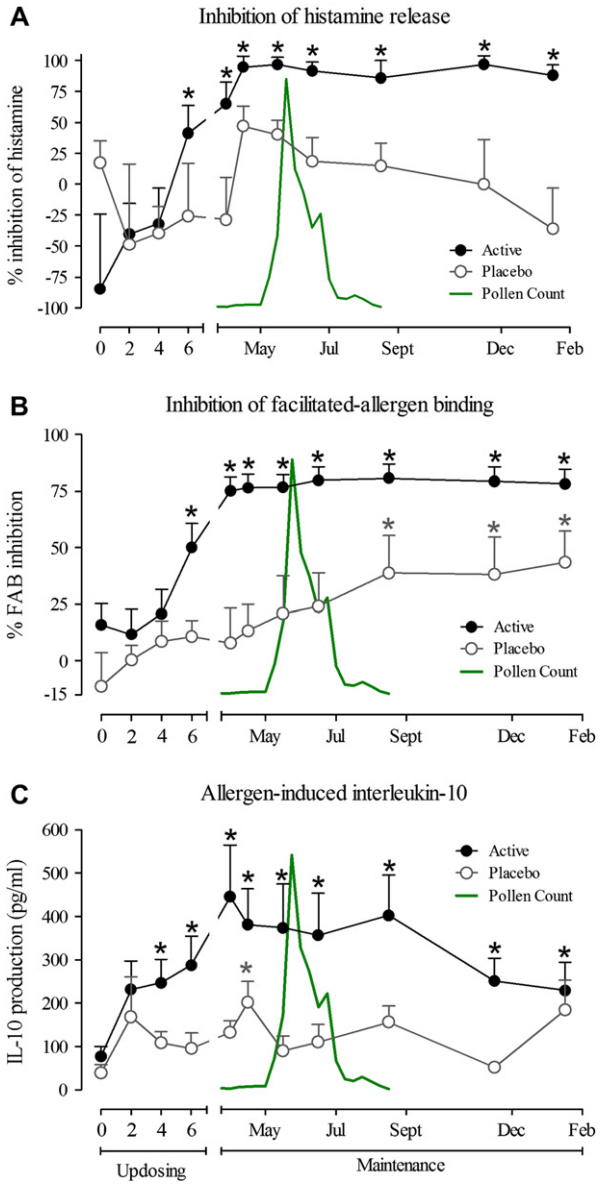

FIG 4. Time-course analysis of serum inhibitory activity and IL-10 production during the first year of grass pollen immunotherapy. Serum was tested for inhibition of FcεRI-mediated allergen-induced basophil histamine release in whole blood, with histamine being measured by means of ELISA (**A**). Serologic inhibitory activity against FcεRII/CD23-dependent IgE-facilitated allergen binding (**B**) was measured by using the IgE-facilitated allergen binding assay *(FAB)*. IL-10 production by PBMCs stimulated with grass pollen allergen was measured by means of ELISA (**C**). Data are normalized to time and analyzed as previously described. *Primary significance from baseline.

group (Fig 3, *B*). Overall, no differences in IgE levels between the active and placebo groups were observed (Fig 3, *C*). However, a transient but significant increase in IgE levels occurred in the active group from study weeks 6 to 10 (end of updosing) and during the pollen season.

Serum inhibitory activity and IL-10 production

Biologic inhibitory activities of sera were measured in assays of allergen- and FcεRI-dependent IgE-mediated basophil histamine release and FcεRII-dependent allergen–IgE binding to B lymphocytes (facilitated allergen binding). Significant inhibition of basophil histamine release was demonstrated at week 6 (baseline, −84.8% ± 60.54%; week 6, 41.3% ± 22.5%; $P < .001$) in the active group (Fig 4, *A*). Binding of grass pollen allergen–IgE complexes to B lymphocytes was also inhibited at week 6 (baseline, 16.0% ± 6.5%; week 6, 50.0% ± 10.0%; $P < .001$), with subjects having received a cumulative dose of 78,100 SQ-U of grass pollen allergen (Fig 4, *B*). In the placebo group no significant changes in basophil histamine release were observed. However, a gradual increase in serum inhibitory activity on allergen–IgE binding occurred in the placebo group, which reached significance at 30 weeks (baseline, −11.17% ± 14.84%; 30 weeks, 38.73% ± 16.56%; $P < .01$). An increase in IL-10 production from

P pratense–stimulated PBMCs was observed in the active group at 4 weeks (246 ± 77 pg/mL) compared with baseline values (76 ± 24 pg/mL, $P < .01$; Fig 4, *C*). This corresponded to a cumulative dose of 18,100 SQ-U of grass pollen allergen. IL-10 production in the placebo group did not change except for a transient but significant increase at week 14.

DISCUSSION

In this study we have examined in detail the time course of clinical desensitization in relation to cellular and humoral immunologic changes during grass pollen immunotherapy. First, immunotherapy resulted in a striking early induction of peripheral IL-10 responses that accompanied suppression of cutaneous allergen–induced late responses. These events were detected as early as 2 to 4 weeks after the first allergen injection, corresponding to a cumulative allergen dose of only 0.9% to 5.4% of the entire dose administered during updosing. It is well established that low-dose pollen immunotherapy is clinically ineffective.[20,21] Therefore we conclude that IL-10 induction and late response suppression *per se* during conventional immunotherapy are not sufficient alone to account for clinical desensitization to grass pollen allergen. Both anti-T-lymphocyte–based (cat peptide immunotherapy and cyclosporin)[22,23] and anti-IgE–based (omalizumab)[24] therapies are associated with inhibition of late responses to intradermal allergen challenge. In this study inhibition of the late response preceded the appearance of serologic inhibitory antibody activity. We therefore speculate that late response suppression during early immunotherapy could reflect the direct inhibitory effects of IL-10 on cell-mediated mechanisms. IL-10 has numerous properties potentially relevant to late response suppression, including inhibition of CD28 costimulatory signaling in T lymphocytes[25] and MHC class II molecule expression.[26] The subsequent induction of antibodies with biologic inhibitory activity against IgE-mediated mechanisms has the potential to consolidate late response inhibition, as well as suppress early responses to allergen exposure.

We report that after induction of IL-10 responses, serum concentrations of allergen-specific IgG4 and IgA antibodies increase by week 12 of immunotherapy, with inhibitory activity in assays of IgE-mediated responses increasing at 6 weeks. Historically, immunotherapy-induced allergen-specific IgG4 concentrations have correlated poorly with clinical responses to treatment.[27-29] We therefore used biologic assays to test the activity of inhibitory antibodies in responses mediated through both FcεRI high-affinity and FcεRII (CD23) low-affinity IgE receptors by using the basophil histamine release assay and the facilitated allergen-binding assay, respectively. We show that immunotherapy can induce serum inhibitory activity effective in both assays, and this result is consistent with previous studies.[10,15] Allergen-specific inhibitory antibodies therefore have the potential to reduce early responses by blocking FcεRI/IgE-dependent mast cell activation and release of preformed mediators. We observe that serum inhibitory activity precedes reductions in early-phase responses, suggesting a causative role of blocking antibodies in this IgE-mediated process. Furthermore, inhibition of FcεRI-dependent mast cell cytokine production and FcεRI- and FcεRII-mediated antigen capture and presentation to T lymphocytes are mechanisms by which inhibitory antibodies could suppress late responses. Allergen-specific IgG also has the further potential to downregulate allergic responses through ligation of inhibitory FcγRIIb receptors on mast cells,[17] basophils,[30] and dendritic cells.[31]

IL-10 was selected as a cellular marker of the immune response because we and others have found induction of this cytokine to be highly reproducible in patients receiving immunotherapy, whereas peripheral changes in TGF-β or T_H1/T_H2 cytokines levels are less consistent.[9] Moreover, there is a possible causal relationship between IL-10 and IgG4, the latter being a marker of humoral change in this study, because IL-10 potentiates IL-4–induced switch recombination from IgM to IgG4 *in vitro*.[32,33] The time course of IgG4 production *in vivo* is consistent with a causative role for IL-10. Alternatively, the delay between these events could be explained by optimal IgG4 production requiring higher allergen doses compared with IL-10 production. We also report an initial increase in IgE levels after 4 weeks of immunotherapy, followed by a decrease to pretreatment levels, which is consistent with other reports.[34] One possibility is that early transient increases in allergen-specific IgE levels are due to induction of plasma cells from existing allergen-specific memory B cells, with later IgG4 and IgA responses reflecting *de novo* B-cell responses. Allergen-specific IgA represents the first line of immunologic defense at mucosal surfaces, and levels are increased after allergen immunotherapy.[12] We recently reported that IgA can induce IL-10 production from monocytes and is associated with the production of local TGF-β expression.[35] In this study allergen-specific IgA levels paralleled those observed for IgG4, suggesting that these antibodies might be regulated by common factors and could alter immunologic reactivity both systemically and at mucosal surfaces.

Surprisingly, we also observed changes in both provocation tests and immunologic outcomes in the placebo group. All subjects underwent repeated intradermal allergen injections to assess changes in early- and late-phase responses, and it is possible that the placebo group was exposed to a tolerizing low dose of allergen (total, 400 SQ-U), which in turn might account for initial changes in late-phase responses. A placebo-controlled study of cat allergen immunotherapy also reported a significant reduction of late-phase cutaneous responses in both the actively treated and placebo groups at 5 weeks after treatment, although precise data are not given to permit a comparison with our data.[36] We observed significant increases in serum inhibitory activity on FcεRII-mediated facilitated allergen binding in the placebo group. In our previous placebo-controlled trial of grass pollen immunotherapy,[10] this increase was not reported, and the only difference in the clinical protocol between these studies was the use of repeated intradermal allergen testing. This suggests that low allergen doses can also influence immunologic parameters. Relatively minor increases in allergen-specific IgG4 levels in the placebo group were restricted to the grass pollen season.[15] Changes in clinical and immunologic parameters caused by repeated intradermal allergen challenge requires further investigation.

We conclude that rapid induction of IL-10 in response to low-dose grass pollen immunotherapy precedes clinical protection and production of inhibitory antibodies. However, IL-10 might contribute to these processes through development of $IgG4^+$ memory B cells. Although further evaluation of large-scale clinical trials is needed, our data suggest that these parameters might be useful to monitor treatment responses or predict efficacy during early stages of immunotherapy.

We thank ALK-Abelló, Hørsholm, Denmark, for supplying allergen extracts for skin testing, immunotherapy, and *in vitro* studies. We also thank Drs Diana Hae-Won Na and Petra Wachholz for expert technical assistance.

Clinical implications: This study establishes the time course of clinical and immunologic markers of tolerance during allergen immunotherapy.

REFERENCES

1. Bauchau V, Durham SR. Epidemiological characterization of the intermittent and persistent types of allergic rhinitis. Allergy 2005;60:350-3.
2. Lockey RF. "ARIA": global guidelines and new forms of allergen immunotherapy. J Allergy Clin Immunol 2001;108:497-9.
3. Plaut M, Valentine MD. Clinical practice. Allergic rhinitis. N Engl J Med 2005; 353:1934-44.
4. 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.
5. Passalacqua G, Durham SR. Allergic rhinitis and its impact on asthma update: allergen immunotherapy. J Allergy Clin Immunol 2007;119:881-91.
6. Calderon MA, Avles B, Jacobson M, Hurwitz B, Sheikh A, Durham S. Allergen injection immunotherapy for seasonal allergic rhinitis. Cochrane Database Syst Rev 2007:CD001936.
7. Varez-Cuesta E, Bousquet J, Canonica GW, Durham SR, Malling HJ, Valovirta E. Standards for practical allergen-specific immunotherapy. Allergy 2006;61(suppl 82): 1-20.
8. 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.
9. Till SJ, Francis JN, Nouri-Aria K, Durham SR. Mechanisms of immunotherapy. J Allergy Clin Immunol 2004;113:1025-34.
10. 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.
11. Francis JN, Till SJ, Durham SR. Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy. J Allergy Clin Immunol 2003;111:1255-61.
12. Jutel M, Akdis M, Budak F, Aebischer-Casaulta C, Wrzyszcz M, Blaser K, et al. IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. Eur J Immunol 2003;33: 1205-14.
13. Royer B, Varadaradjalou S, Saas P, Guillosson JJ, Kantelip JP, Arock M. Inhibition of IgE-induced activation of human mast cells by IL-10. Clin Exp Allergy 2001;31: 694-704.
14. Takanaski S, Nonaka R, Xing Z, O'Byrne P, Dolovich J, Jordana M. Interleukin 10 inhibits lipopolysaccharide-induced survival and cytokine production by human peripheral blood eosinophils. J Exp Med 1994;180:711-5.
15. Mothes N, Heinzkill M, Drachenberg KJ, Sperr WR, Krauth MT, Majlesi Y, et al. Allergen-specific immunotherapy with a monophosphoryl lipid A-adjuvanted vaccine: reduced seasonally boosted immunoglobulin E production and inhibition of basophil histamine release by therapy-induced blocking antibodies. Clin Exp Allergy 2003;33:1198-208.
16. 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.
17. Kepley CL, Taghavi S, Mackay G, Zhu D, Morel PA, Zhang K, et al. Co-aggregation of FcgammaRII with FcepsilonRI on human mast cells inhibits antigen-induced secretion and involves SHIP-Grb2-Dok complexes. J Biol Chem 2004; 279:35139-49.
18. 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.
19. Klunker S, Saggar 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.
20. Franklin W, Lowell FC. Comparison of two dosages of ragweed extract in the treatment of pollenosis. JAMA 1967;201:915-7.
21. Van Metre TE Jr, Adkinson NF Jr, Lichtenstein LM, Mardiney MR Jr, Norman PS Jr, Rosenberg GL, et al. A controlled study of the effectiveness of the Rinkel method of immunotherapy for ragweed pollen hay fever. J Allergy Clin Immunol 1980;65:288-97.
22. Oldfield WL, Larche M, Kay AB. Effect of T-cell peptides derived from Fel d 1 on allergic reactions and cytokine production in patients sensitive to cats: a randomised controlled trial. Lancet 2002;360:47-53.
23. Sihra BS, Kon OM, Durham SR, Walker S, Barnes NC, Kay AB. Effect of cyclosporin A on the allergen-induced late asthmatic reaction. Thorax 1997;52: 447-52.
24. Ong YE, Menzies-Gow A, Barkans J, Benyahia F, Ou TT, Ying S, et al. Anti-IgE (omalizumab) inhibits late-phase reactions and inflammatory cells after repeat skin allergen challenge. J Allergy Clin Immunol 2005;116:558-64.
25. Schandene L, Alonso-Vega C, Willems F, Gerard C, Delvaux A, Velu T, et al. B7/CD28-dependent IL-5 production by human resting T cells is inhibited by IL-10. J Immunol 1994;152:4368-74.
26. Akdis CA, Joss A, Akdis M, Faith A, Blaser K. A molecular basis for T cell suppression by IL-10: CD28-associated IL-10 receptor inhibits CD28 tyrosine phosphorylation and phosphatidylinositol 3-kinase binding. FASEB J 2000;14: 1666-8.
27. 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.
28. 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.
29. Djurup R, Osterballe O. IgG subclass antibody response in grass pollen-allergic patients undergoing specific immunotherapy. Prognostic value of serum IgG subclass antibody levels early in immunotherapy. Allergy 1984;39:433-41.
30. Kepley CL, Cambier JC, Morel PA, Lujan D, Ortega E, Wilson BS, et al. Negative regulation of FcepsilonRI signaling by FcgammaRII costimulation in human blood basophils. J Allergy Clin Immunol 2000;106:337-48.
31. Boruchov AM, Heller G, Veri MC, Bonvini E, Ravetch JV, Young JW. Activating and inhibitory IgG Fc receptors on human DCs mediate opposing functions. J Clin Invest 2005;115:2914-23.
32. Jeannin P, Lecoanet S, Delneste Y, Gauchat JF, Bonnefoy JY. IgE versus IgG4 production can be differentially regulated by IL-10. J Immunol 1998;160:3555-61.
33. Vercelli D, De Monte L, Monticelli S, Di Bartolo C, Agresti A. To E or not to E? Can an IL-4-induced B cell choose between IgE and IgG4? Int Arch Allergy Immunol 1998;116:1-4.
34. Creticos PS, Van Metre TE, Mardiney MR, Rosenberg GL, Norman PS, Adkinson NF Jr. Dose response of IgE and IgG antibodies during ragweed immunotherapy. J Allergy Clin Immunol 1984;73:94-104.
35. Pilette C, Nouri-Aria KT, Jacobson MR, Wilcock LK, Detry B, Walker SM, et al. Grass pollen immunotherapy induces an allergen-specific IgA2 antibody response associated with mucosal TGF-beta expression. J Immunol 2007;178:4658-66.
36. Nanda A, O'Connor M, Anand M, Dreskin SC, Zhang L, Hines B, et al. Dose dependence and time course of the immunologic response to administration of standardized cat allergen extract. J Allergy Clin Immunol 2004;114:1339-44.

METHODS

Immunotherapy

Patients with seasonal allergic rhinitis were selected on the basis of a history of poor disease control in previous years, despite treatment with antihistamines, corticosteroid nasal sprays, and/or cromoglycate eye drops. Poor disease control constituted troublesome symptoms, impairment of sleep or social functioning (eg, leisure or work activities), or both. These patients were therefore classified as having moderate/severe symptoms (despite optimal pharmacotherapy) according to the Allergic Rhinitis and its Impact on Asthma classification of rhinitis. A standardized, aluminum hydroxide–adsorbed, depot timothy grass pollen (whole extract) vaccine (Alutard SQ, ALK-Abelló) was used for subcutaneous injection immunotherapy. Injections were administered according to a modified cluster regimen (Table E1). In brief, in the first 2 months, patients underwent weekly visits for updosing, receiving 2 injections separated by 1 hour containing increasing quantities of grass pollen vaccine. Thereafter, at each monthly visit, patients were administered with a 1-mL maintenance injection containing 100,000 SQ-U (containing 20 μg of the major grass allergen Phl p 5), except during the pollen season, when the maintenance dose was reduced by 40%. Placebo injections consisted of identical vials of diluent, including aluminum hydroxide and 0.01 mg/mL histamine. All injections were administered in the upper arm, and patients were observed for 1 hour after each injection. Patients commenced induction therapy over a period of 6 to 8 weeks, and all patients were receiving maintenance injections before the start of the grass pollen season. In general, immunotherapy was well tolerated, with the occurrence of only transient minor local swellings at injection sites that required no treatment. Two participants had a rapid onset of general urticarial rash and wheezing that came on within 15 minutes of injection. The patients received adrenaline, 0.5 mL of a 1:1000 solution, intramuscularly as a precaution, with rapid resolution of symptoms. Both patients continued in the trial at a modified monthly maintenance dose of 10,000 SQ-U. All study participants had access to antihistamines and topical cromoglycate eye drops during the pollen season, which were discontinued at least 48 hours before skin testing or venesection.

Clinical tests

On completion of the study, all patients underwent conjunctival provocation testing by instilling half-log (approximately 3-fold) incremental concentrations of grass pollen extract (100-100,000 SQ-U/mL) into alternate eyes at 10-minute intervals. Immediate conjunctival sensitivity was recorded as the dose that induced a minimum of 2 of 4 symptoms (itching, redness, tears, or swelling). If there was no response at the highest concentration of allergen tested (100,000 SQ-U/mL), the outcome was arbitrarily assigned a value of 300,000 SQ-U/mL.

Pollen counts

Daily pollen counts (grains per cubic meter) for the London region were received courtesy of Dr Jean Emberlin (UK Pollen Centre, University of Worcester).

Immunologic tests

For serologic assays, 10 mL of peripheral blood was collected in sterile glass tubes (Vacutainer; Becton Dickinson, Mountain View, Calif) and allowed to coagulate. For histamine release experiments, whole blood from an atopic donor with serum levels of *P pratense*–specific IgE of greater than 100 U/L was incubated with 1 pg/mL *P pratense* grass pollen extract in the presence or absence of sera from immunotherapy-treated patients at 37°C for 1 hour, followed by a further 10-minute incubation on ice. Histamine concentrations in cell-free supernatants were measured by means of ELISA (IBL), and results were expressed as percentage inhibition by immunotherapy-treated serum compared with control serum. The limit of detection for the assay was 0.2 ng/mL.

TABLE E1. Clinical protocol of immunotherapy study

Month	Visit	Injection no.	Dose (SQ-U)	Cumulative dose (SQ-U)	Skin testing and venesection*
					←
1	Week 0	1	100		
		2	1,000	1,100	
	Week 1	3	1,000		
		4	1,000	3,100	
					←
	Week 2	5	2,000		
		6	3,000	8,100	
	Week 3	7	5,000		
		8	5,000	18,100	
					←
2	Week 4	9	10,000		
		10	10,000	38,100	
	Week 5	11	20,000		
		12	20,000	78,100	
					←
	Week 6	13	30,000		
		14	30,000	138,100	
					←
	Week 7	15	100,000	238,100	
		16	100,000	338,100	
					←
3		17	60,000	438,100	
					←
4		18	60,000	498,100	
					←
5		19	60,000	558,100	
					←
6		20	100,000	618,100	
7		21	100,000	718,100	
8		22	100,000	818,100	
					←
9		23	100,000	918,100	
10		24	100,000	1,018,100	
11		25	100,000	1,118,100	
					←
12		26	100,000	1,218,100	

100,000 SQ-U = 20 μg of the Phl p 5 major allergen.
*Venesection and intradermal skin testing performed 24 hours before immunotherapy injection.