

Repeated low-dose intradermal allergen injection suppresses allergen-induced cutaneous late responses

Giuseppina Rotiroti, MD,^a Mohamed Shamji, PhD,^{a,c} Stephen R. Durham, MD, FRCP,^{a,c} and Stephen J. Till, MRCP, PhD^{a,b,c} London, United Kingdom

Background: Subcutaneous immunotherapy with high-dose grass pollen was first described more than 100 years ago. This treatment suppresses allergen-induced cutaneous late responses, with lesser effects on early responses. In contrast, low-dose subcutaneous immunotherapy has not shown clinical benefit. Uncontrolled reports from the early 20th century describe low-dose allergen inoculation directly into the dermis, an immunologically active area containing abundant dendritic cells and lymphatics.

Objective: We sought to investigate the effect of low-dose intradermal grass pollen administration on cutaneous reactivity to allergen.

Methods: Thirty adults sensitized to grass and tree pollens were randomized to receive (1) 6 repeat intradermal injections at 2-week intervals of grass pollen extract (estimated 7 ng of the major grass allergen Phl p 5 per injection), (2) 2 intradermal injections separated by 10 weeks, or (3) a single intradermal injection at 10 weeks. At the end of the study, cutaneous early and late responses were measured after double-blind intradermal injection with grass and birch pollen.

Results: Participants who received 6 fortnightly intradermal grass pollen injections had markedly smaller cutaneous late responses to grass pollen than control subjects who received 2 injections separated by 10 weeks ($P < .01$) or a single injection ($P < .001$) and showed induction of grass pollen-specific IgG antibodies. Suppression was observed whether late responses were measured on the arms or the back. However, early responses were equivalent in all groups.

Conclusion: Low-dose intradermal allergen, like conventional subcutaneous high-dose immunotherapy, suppresses allergen-induced cutaneous late responses in a manner that is

allergen specific, systemic, and associated with induction of IgG antibodies. (*J Allergy Clin Immunol* 2012;■■■:■■■-■■■.)

Key words: Tolerance, immunotherapy, intradermal, grass, late response, IgE

Subcutaneous allergen immunotherapy involves the repeated injection of allergen extracts to IgE-sensitized allergic subjects to induce clinical and immunologic tolerance. Noon¹ published his seminal article on prophylactic inoculation of grass pollen for hay fever in the *Lancet* a century ago, long before the availability of effective antiallergy drugs. William Frankland, also from St Mary's Hospital in London, published the first successful double-blind trial of grass pollen immunotherapy in the same journal in 1954.² Immunotherapy traditionally involves administration of high allergen doses and carries the risk of systemic side effects,³ whereas the use of low doses of subcutaneous allergen, as described in historical studies,⁴ is highly controversial and has not shown benefit in small clinical trials.⁵ However, uncontrolled reports dating from the early 20th century suggest that low doses of allergen administered through the intradermal route might be clinically effective. For example, in 1926, Phillips⁶ described a series of 29 patients to whom he administered intradermal grass pollen extract in Arizona, which he described as being "monotonously successful."

In this study we show that up to 6 fortnightly administrations of very low doses of grass pollen allergen extract (cumulative doses 2000-fold less than usually used for subcutaneous treatment), when administered intradermally, might induce a substantial time- and dose-dependent suppression of the late cutaneous response to the offending allergen. This desensitizing effect is allergen specific and accompanied by an increase in systemic allergen-specific IgG antibodies, together with increases in serum inhibitory activity against IgE allergen binding. These data invite a reconsideration of low-dose intradermal allergen as a potentially effective alternative to the usual subcutaneous route.

METHODS

Recruitment and characterization of study subjects

Thirty adults with a history of seasonal allergic rhinitis for a minimum of 2 years during the United Kingdom tree and grass pollen seasons (March/April and June/July, respectively) were recruited from the Royal Brompton Hospital Allergy Clinic or by advertisement in the local press. All subjects underwent subsequent clinical screening outside the pollen season, including skin prick testing to a standard range of common inhalant allergens (*Dermatophagoides pteronyssinus*, 6-grass pollen mix, timothy grass, 3-tree pollen mix, silver birch, *Aspergillus* species, *Alternaria* species, *Cladosporium* species, cat dander, horse dander, and dog dander) in the presence of positive (histamine) and negative (diluent) controls. Participants in the study were required to show dual sensitization to timothy grass (*Phleum pratense*) and silver birch (*Betula verrucosa*) pollen, as evidenced by positive skin test responses (wheal diameter ≥ 3 mm larger than that elicited by the negative diluent control: Soluprick;

From ^aAllergy and Clinical Immunology, National Heart and Lung Institute, Imperial College London; ^bAsthma, Allergy & Lung Biology, King's College London; and ^cMRC & Asthma UK Centre in Allergic Mechanisms of Asthma.

S.J.T. was supported by a Clinician Scientist Fellowship from the Health Foundation and Academy of Medical Sciences. This study was also supported by the Royal Brompton and Harefield Hospitals Charitable Trust.

Disclosure of potential conflict of interest: G. Rotiroti has received travel expenses from ALK-Abelló, GlaxoSmithKline, and Allergy Therapeutics. S. R. Durham has received consultancy fees from ALK-Abelló, Merck, Circassia, GlaxoSmithKline, and Boehringer Ingelheim; has provided expert testimony for Weil; has received research support from ALK-Abelló and Merck; and has received lecture fees from Merck, ALK-Abelló, and GlaxoSmithKline. S. J. Till has received research support from ALK-Abelló. M. Shamji declares that he has no relevant conflicts of interest.

Received for publication October 12, 2011; revised April 30, 2012; accepted for publication June 22, 2012.

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

0091-6749/\$36.00

© 2012 American Academy of Allergy, Asthma & Immunology

<http://dx.doi.org/10.1016/j.jaci.2012.06.052>

Abbreviations used

BU: Biological units
 DC: Dendritic cell
 FAB: Facilitated allergen binding
 SQ-U: Standardized quality units

ALK-Abelló, Hørsholm, Denmark). Subjects were excluded if they had symptomatic allergic rhinitis or asthma requiring regular pharmacotherapy outside March–August, had received immunotherapy with grass or birch pollen allergen within the previous 10 years, were taking prednisolone or other immunosuppressive therapies, or were taking β -blockers. The study was approved by the Ethics Committee of the Royal Brompton and Harefield NHS Trust, and informed written consent was obtained before participation.

Study design

The design of the study is summarized in Fig 1. Subjects were stratified on the basis of skin test response size in response to grass pollen before being randomly allocated to one of 3 study arms. Subjects assigned to group A underwent repeated intradermal injection with *P pratense* pollen extract fortnightly for 6 consecutive visits over a total period of 10 weeks. Group A subjects also received control intradermal injections of *B verrucosa* extract at the first and last study visits only (visits 1 and 6) to investigate the allergen specificity of any changes in the size of responses to *P pratense*. Subjects in group B underwent intradermal injection with both *P pratense* (grass) and *B verrucosa* (birch) pollen extracts at the first and last visits only (visits 1 and 6). The reason for including group B was to test whether a single intradermal injection administered 10 weeks previously could account for any curtailment of late responses seen in group A at the final visit (visit 6), as opposed to the effects of repetitive intradermal injection over the same time period. Group C subjects underwent intradermal injection with *P pratense* and *B verrucosa* at the last study visit only (ie, visit 6). Single batches of grass and birch pollen allergen extract were stored as frozen aliquots and used for all intradermal tests through the study after a single freeze-thaw cycle to minimize experimental variation. Group C subjects were therefore included as a cross-sectional control group to exclude the possibility of allergen extract degradation in the event of late-response inhibition in both groups A and B at visit 6. Serum was collected from all participants at the time points indicated in Fig 1. The primary outcome in this study was the size of the grass pollen–induced late response in group A versus group B at visit 6.

Intradermal allergen provocation

Intradermal injections were administered on the extensor aspect of the forearm. Injections were spaced at least 10 cm apart to avoid overlap and minimize interaction between adjacent tests. Grass and birch pollen extracts (Aquagen SQ, ALK-Abelló) were injected in 20- μ L volumes. Intradermal injections were all administered with a 29-gauge insulin syringe (Micro-Fine; Becton Dickinson, Franklin Lakes, NJ) filled with 20 μ L of solution containing 0.1, 1.0, and 10 biological units (BU) of grass or birch pollen allergen extract (equivalent to approximately 0.33, 3.3, and 33 of manufacturer's standardized quality units [SQ-U]; Aquagen SQ, ALK-Abelló) or diluent as a negative control. For grass and birch pollen extracts, 10 BU is estimated to contain 7 ng of the Phl p 5 and 4 ng of Bet v 1 major allergens, respectively (data supplied by manufacturer). The entire 20- μ L volume was injected intradermally to raise a visible bleb. Successful intradermal injection of allergen was confirmed each time by the presence of a wheal (ie, the early response) at the injection site after 20 minutes. Grass and birch pollen intradermal injections were randomized and administered double-blind at both the first visit (visit 1: groups A and B) and the final visit (visit 6: all groups), and therefore neither investigator nor study subject was aware of the content of a given injection. At the end of the study, intradermal injections with 10 BU of grass pollen extract were also performed on the backs of subjects from groups A and C to determine whether late-response suppression was systemic. Early

responses were measured 20 minutes after injection by documenting the size of the resulting skin wheal. Late skin responses were identified and measured according to the perimeter of redness, swelling, edema, and induration at 24 hours. All measurements were performed double-blind to the allergen used for testing by a single investigator and subsequently transcribed onto paper for response size quantification by means of computer planimetry.

Antibody measurements and facilitated antigen binding assay

P pratense–specific IgG, IgG₁, and IgG₄ concentrations were measured by means of ELISA (coating allergen concentration, 5 mg/mL; 1:20 dilution of sera; detection with biotinylated anti-human IgG, IgG₁, or IgG₄ mAb; BD PharMingen, San Diego, Calif). Results were expressed as arbitrary units compared with the calibrator serum obtained from patients receiving long-term grass pollen immunotherapy. The IgE-dependent facilitated allergen binding (IgE-FAB) assay was performed as previously described⁷ to assess the inhibitory bioactivity of sera against allergen-IgE complex formation.

Briefly, allergen-IgE complexes were formed by incubating 20 mL of sera containing high concentrations of *P pratense*–specific IgE (>100 IU/mL) with 1 *P pratense* extract (0.3 μ g/mL) in the presence or absence of 20 mL of sera from study participants 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 by means of flow cytometry. Surface-bound IgE-allergen complexes were detected directly with a fluorescently conjugated anti-IgE antibody.

Statistics

Data were analyzed by using GraphPad Prism software (GraphPad Software, La Jolla, Calif). The D'Agostino and Pearson test was used to establish data normality, and parametric statistical tests were subsequently used throughout. Comparisons among the 3 groups at visit 6 were performed by using 1-way ANOVA. Where results were significant, posttest individual comparisons were performed between groups A and B or groups A and C by using the Bonferroni multiple comparison test. Analysis of data collected over more than 2 time points was performed by using repeated measures ANOVA, followed by posttest comparison with visit 1 (baseline) by using the Bonferroni multiple comparison test. Otherwise, data were compared with unpaired or paired Student *t* tests, as appropriate. A *P* value of less than .05 was considered significant.

RESULTS

Thirty participants were randomized to the 3 study groups (groups A, B, and C). These groups were matched for age, sex, and skin prick test response size to grass and birch pollen (Table I). One subject withdrew from group C for reasons unrelated to the study and was not replaced. There were no instances of systemic allergic reactions after intradermal injections.

Forearm cutaneous late responses measured 24 hours after intradermal injection of 10 BU of grass (*P pratense*) pollen extract were significantly different among the 3 groups at visit 6 ($P < .001$, Fig 2). Subsequent comparisons showed that response sizes in group A (mean $\pm$ SE area, 801 ± 275 mm²; $n = 10$) were lower than in both group B (6560 ± 1587 mm², $n = 10$, $P < .01$) and group C (8178 ± 982 mm², $n = 9$, $P < .001$). In contrast, cutaneous late responses to 10 BU of birch (*B verrucosa*) pollen extract were equivalent among groups A (mean $\pm$ SE area, 3434 ± 892 mm²; $n = 10$), B (3816 ± 808 mm², $n = 10$), and C (4452 ± 1236 mm², $n = 9$; $P = .78$, Fig 2). In contrast to the late response, early responses measured 20 minutes after intradermal injection of 10 BU of grass pollen at visit 6 were equivalent in groups A (287 ± 32 mm², $n = 10$), B (396 ± 71 mm²), and C

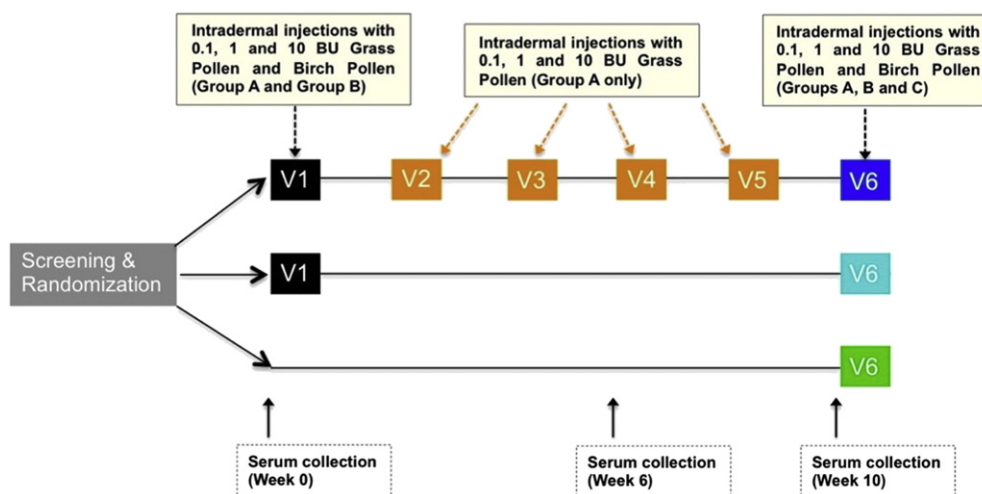


FIG 1. Outline of study design. At each visit, indicated intradermal injections with 0.1, 1, and 10 BU of grass (*P pratense*) pollen extract were performed. The early and late responses were measured after 20 minutes and 24 hours, respectively.

TABLE I. Clinical characteristics of participants

	Group A (n = 10)	Group B (n = 10)	Group C (n = 9)	P value*
Male/female sex	4/6	3/7	2/8	.65
Age (y)	32.8 ± 2.9	36.7 ± 3.3	35.6 ± 3.0	.65
SPT, grass	9.7 ± 0.8	8.9 ± 1.3	10.0 ± 1.0	.73
SPT, birch	6.2 ± 0.4	6.5 ± 0.7	6.1 ± 0.7	.86

Data are shown as means ± SEs. One subject subsequently withdrew from group C for reasons unrelated to the study.

SPT, Skin prick test–induced wheal size (in millimeters).

*Comparisons among the 3 groups were done with the χ^2 test (sex) or 1-way ANOVA (all other parameters).

(345 ± 58 mm², $P = .40$). Similarly, no differences were seen between these groups in early responses to 10 BU of intradermal birch pollen (groups A, B, and C: 315 ± 25, 454 ± 86, and 315 ± 41 mm², respectively; $P = .16$, Fig 3).

Sixteen participants from groups A and C were recalled after visit 6 for intradermal grass pollen (10 BU) injections on the back to investigate whether suppression of allergen-induced late responses occurred at a distant site. Responses were smaller in size than those observed on the forearm. Nevertheless, late-response sizes in group A were suppressed compared with those in group C (198 ± 50 and 956 ± 190 mm², respectively; $n = 8$ both groups; $P < .005$).

In group A the size of the cutaneous late response to intradermal injection of 0.1, 1.0, and 10 BU of grass pollen administered simultaneously in different areas of the forearm was measured at visit 1 (baseline) and compared at visits 2, 3, 4, 5, and 6. Repeated intradermal injections were associated with longitudinal reductions in the size of late responses ($P < .0001$ for each of 0.1, 1.0, and 10 BU; Fig 4). Within each dose, reductions in the size of late responses achieved statistical significance as early as visit 2. By visit 6, late responses to 0.1, 1.0, and 10 BU were inhibited by 92.4% ± 2.4%, 96.0% ± 1.0%, and 92.6% ± 2.0% (mean inhibition ± SE compared with baseline visit 1 size), respectively. For each allergen dose, the suppressive effect of repeated injections on late-response size by visit 6 was considerably greater than the effect recorded on early-response size (mean ± SE inhibition

compared with baseline visit 1: 0.1 BU, 92.4% ± 2.4% [late response] vs 30.4% ± 5.1% [early response; $P < .0001$]; 1.0 BU, 96.0% ± 1.0% [late response] vs 4.0% ± 15.2% [early response; $P < .0005$]; and 10 BU, 92.6% ± 2.0% [late response] vs 29.1% ± 6.9% [early response; $P < .0001$]; see Fig E1 in this article's Online Repository at www.jacionline.org).

Grass-specific total IgG, IgG₁, and IgG₄ titers were measured in sera to evaluate the possible effects of repeated intradermal injection with grass pollen on underlying immune responses. In group A IgG (ie, all isotypes) titers were increased compared with baseline values when measured before visit 4 (6 weeks after first injection) or before visit 6 (10 weeks after first injection; $P < .005$ at both time points, Fig 5). Changes in absolute levels of allergen-specific IgG₁ and IgG₄ isotypes did not achieve statistical significance, although when re-expressed as fold increase, IgG₁ levels did increase in group A by the time of visit 6 (2.4-fold increase ± 0.5 vs visit 1: $P = .02$). No changes in antibody levels were observed in groups B or C during the 10-week period of the study. The inhibitory bioactivity of sera was further examined by measuring their ability to block IgE–grass pollen complex binding in a cellular assay. Sera from group A subjects collected before visit 6 (week 10) showed an increase in inhibition of IgE–allergen complex binding to B cells when compared with sera collected before visit 4 (week 6; $P < .01$, Fig 5). Changes in inhibitory activity of sera were not observed in groups B or C during the same study period.

DISCUSSION

Prophylactic inoculation with grass pollen for the treatment of hay fever was first described in 1911.¹ The conventional approach, which has been validated in numerous clinical trials,⁸ involves the regular subcutaneous administration of allergen extracts at high doses (typically microgram quantities of allergens).⁹ However, a form of low-dose immunotherapy (typically involving administration of nanogram quantities of allergens) was also historically practiced according to the so-called Rinkel method.⁴ It is often assumed that the Rinkel method involved intradermal allergen immunotherapy. However, close inspection of Rinkel's original publications reveals that intradermal injections

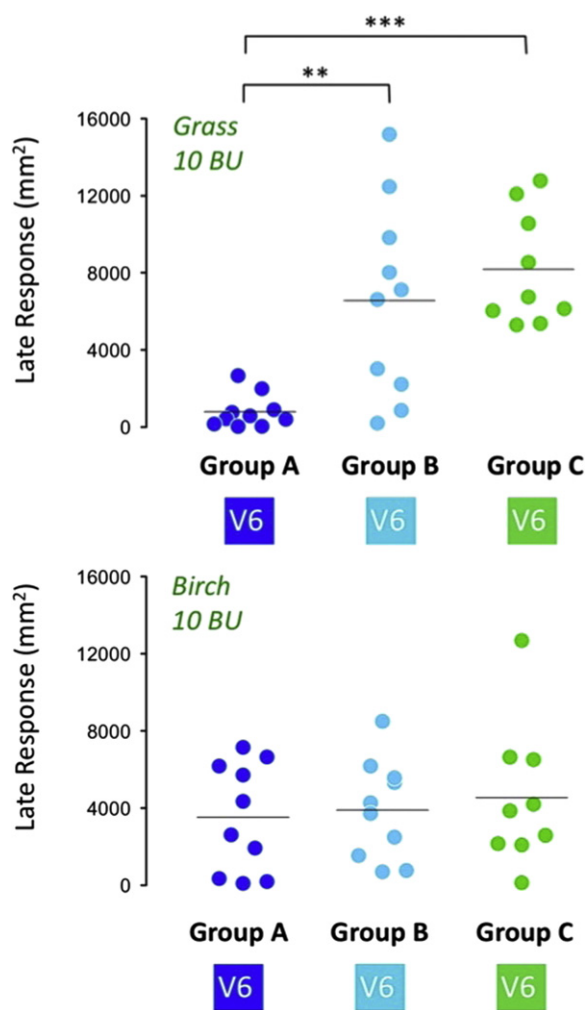


FIG 2. Cutaneous late responses (24 hours) to intradermal injection of 10 BU of grass (*P pratense*) and birch (*B verrucosa*) pollen extract at the final visit (visit 6). Bars indicate mean values. Comparisons among the 3 groups at visit 6 were performed by using 1-way ANOVA with posttest individual comparisons performed with the Bonferroni multiple comparison test. ** $P < .01$ and *** $P < .001$.

were used for titration testing to identify a starting allergen dilution, which was subsequently administered by using “the ordinary subcutaneous method”.⁴ Rinkel’s method was subsequently examined in a later randomized controlled trial by Van Metre et al⁵ and reported to be ineffective. In that study allergen dilution was again determined by using “intracutaneous testing,” but therapeutic administration involved “subcutaneous injections.” However, there are limited uncontrolled historical reports of true intradermal allergen immunotherapy that are necessarily limited to smaller doses than tolerated by the subcutaneous route because of prominence of local reactions. In 1926, Phillips,¹⁰ working in Arizona, published a preliminary account of this treatment in 29 patients,⁶ which was extended to 322 patients by 1933,¹⁰ more than 90% of whom obtained “satisfactory relief.” To our knowledge, this phenomenon of intradermal injection with low-dose allergen extracts has not been readdressed since the work of Phillips.¹⁰

In a previous clinical study of allergen immunotherapy, we unexpectedly observed a tendency in placebo-treated participants

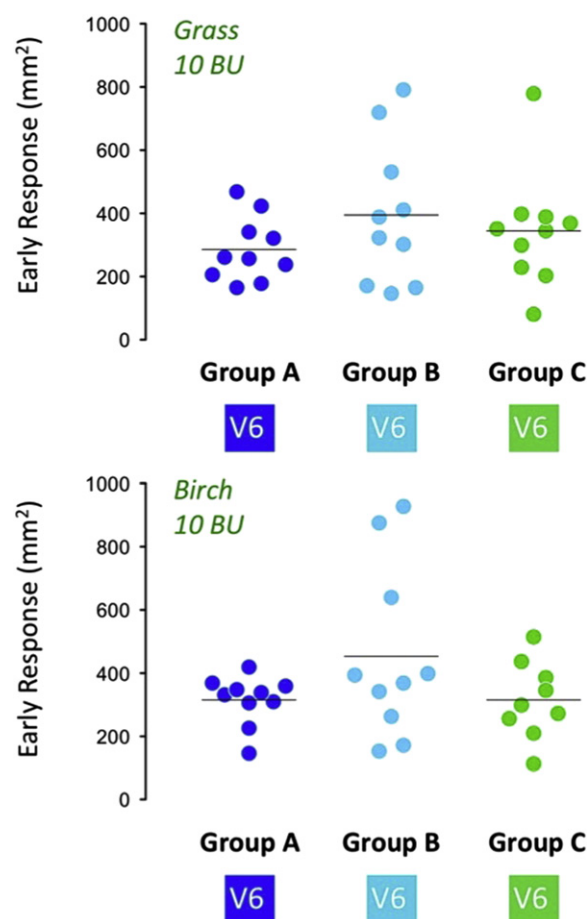


FIG 3. Cutaneous early responses (20 minutes) to intradermal injection of 10 BU of grass (*P pratense*) and birch (*B verrucosa*) pollen extract at the final visit (visit 6). Bars indicate mean values. Comparisons among the 3 groups at visit 6 were performed by using 1-way ANOVA.

for the size of the cutaneous late response induced by intradermal allergen challenge to diminish when repeated on multiple occasions.⁷ The present study, which was prospectively designed to investigate this phenomenon under controlled conditions, confirms that repeated low-dose intradermal injection of grass pollen allergen is associated with suppression of the cutaneous late response elicited in response to this provocation. This effect was allergen specific, as evidenced by the lack of effect on the birch pollen responses, and systemic, as evidenced by the suppression of the late responses at a distal site. The effect was also profound, with more than 90% inhibition of late responses by the end of the 10-week study period.

The cumulative dose of allergen administered intradermally over 10 weeks equated to approximately 50 ng of the major group 5 allergen of timothy grass pollen (Phl p 5). This is 2000-fold less than we previously administered subcutaneously over the same time period as part of a cluster grass pollen immunotherapy protocol and that was associated with comparable late-response suppression.⁷ In a previous study from our group, a grass pollen extract produced by the same manufacturer as drops for sublingual use delivered 20,000-fold more group 5 allergen over a 10-week period than was administered in this study but was associated with a lesser inhibition of late responses, an approximate 40% reduction compared with placebo.¹¹

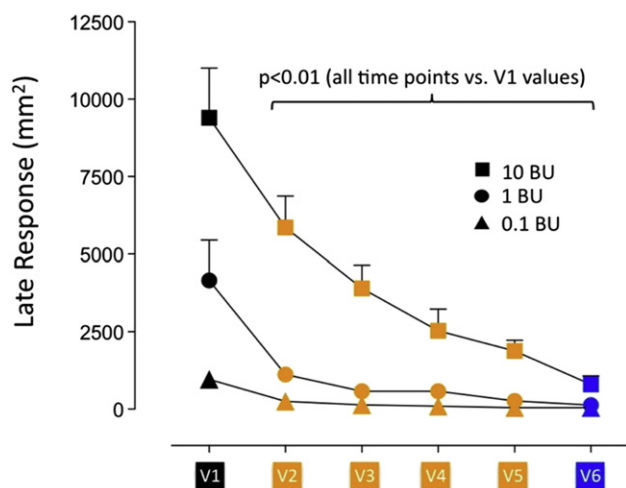


FIG 4. Time course of suppression of allergen-induced late responses in group A subjects. Data shown represent the mean $\pm$ SEM area of late responses measured 24 hours after intradermal injection of 0.1, 1.0, or 10 BU of *P pratense* grass pollen extract at 6 repeat visits separated by 2-week intervals. For each allergen dose, significance was assessed by using repeated measures ANOVA, followed by posttest comparison with visit 1 (baseline) using the Bonferroni multiple comparison test.

A strength of this study is that all subjects were stratified according to skin test response size before randomization into intervention or control groups. Patients with dual grass/birch sensitivity were also enrolled so that measurements in response to grass and birch at visit 6 could be randomized and blinded with respect to investigator and study subjects. Thus the primary outcome (grass pollen-induced late response size in group A vs group B at visit 6/week 10) was measured under double-blind conditions. Group B subjects controlled for the effect of a single previous intradermal allergen injection at the start of the study, as opposed to repeated injections over the same time frame. Group C control subjects were included as a precaution to exclude the possibility of allergen extract degradation in the event of reduced late responses to grass being observed in both groups A and B at visit 6.

The allergen-induced late response has been extensively used as a human experimental model for studying mechanisms of the chronic inflammatory component of allergic inflammation and the influence of pharmacotherapy and immunotherapy. The late-phase response is thought to be partly dependent on local infiltration and activation of proinflammatory T_H cells, including T_H2 cells, which are prominent during such reactions.^{12,13} However, cutaneous late responses also have an IgE-dependent component, as evidenced by partial suppression with omalizumab,¹⁴ suggesting priming for late responses by means of activation of skin-resident mast cells, activation of recruited basophils, or IgE-dependent T-cell activation though facilitation of binding to antigen-presenting cells.¹⁵ Subcutaneous immunotherapy with inhalant and wasp venom allergens,^{7,13,16-18} as well as sublingual immunotherapy,¹¹ is associated with suppression of allergen-induced late responses. Effects on allergen-induced early responses, similar to the findings from this study, appear less marked.⁷ Consequently, cutaneous late-response suppression has been used in many studies as an experimental model to investigate immunotherapy-induced immunologic mechanisms or as a biomarker of such changes. However, the surrogacy of the late response for clinical efficacy and the allergen specificity of

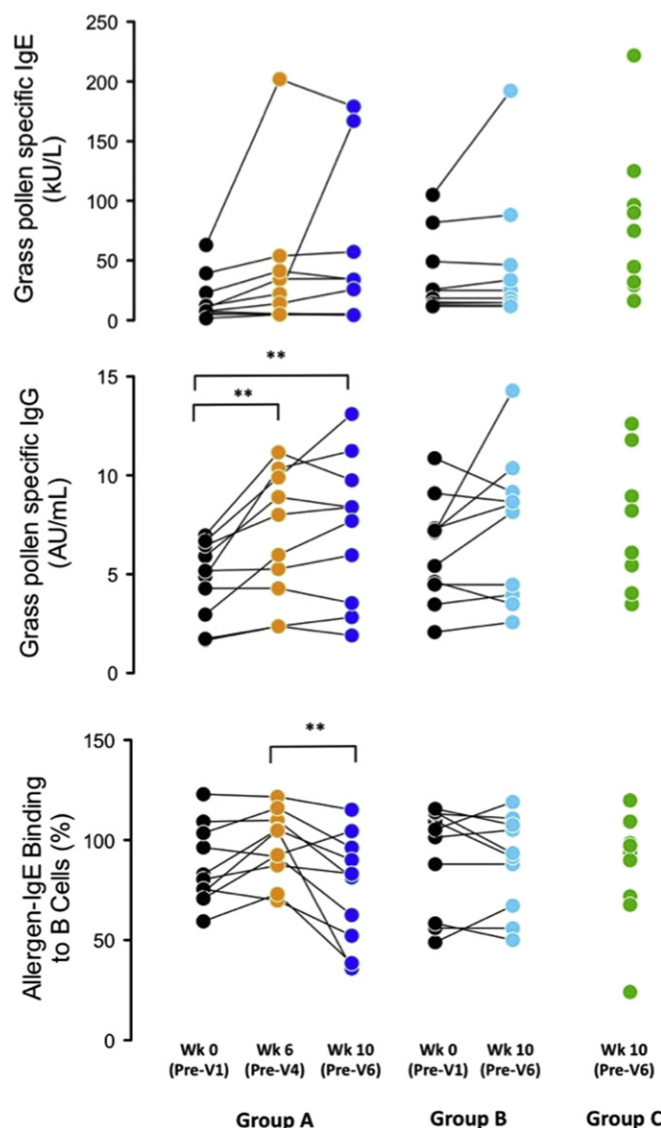


FIG 5. Effect of repeated intradermal injection with *P pratense* grass pollen extract on allergen-specific IgE and IgG titers together with inhibitory activity against IgE-grass pollen complex binding in the FAB assays. Sera were collected before intradermal injection at the visit shown and analyzed by using ELISA. For group A, significance was assessed by using repeated measures ANOVA, followed by posttest comparisons of different time points using the Bonferroni multiple comparison test. $^{**}P < .01$. For group B, baseline and week 10 measurements were compared with the paired Student *t* test.

immunotherapy-induced late-response suppression have not been established. In contrast, the effect of repeated intradermal low-dose allergen exposure on allergen-induced late responses has not been examined.

A previous report investigated the progressive suppression of delayed local reactions to repeated bee stings in beekeepers.¹⁹ Although stings likely involve deposition of antigen within the dermis, this phenomenon is distinct from that described in the present study. First, the beekeepers were classified as "nonallergic," and the size of local reactions was far smaller than the late responses observed here in response to intradermal grass pollen allergen. Second, stings contain considerably more antigen than a standard intradermal allergen injection and are roughly comparable with a

subcutaneous immunotherapy dose. For example, a typical bee sting contains 50 μg of venom, whereas the highest dose of bee venom recommended for intradermal testing is in the range of 30 ng (of which approximately 3 ng represents the major allergen Api m 1).^{20,21} In comparison, a typical grass pollen subcutaneous immunotherapy maintenance dose contains 20 μg of Phl p 5 in comparison with the 7 ng administered intradermally in this study.⁹

An experimental cat peptide immunotherapy vaccine has also been administered through the intradermal route. However, the dose of antigen administered in such studies also greatly exceeds that which we administered intradermally. For example, cat peptides were generally administered in nanomolar quantities,²² whereas 7 ng of Phl p 5 corresponds to approximately 0.2 picomoles of allergen.

The intradermal route is a well-established concept in the field of prophylactic vaccination for infectious diseases. For example, the BCG vaccine has been administered intradermally for more than half a century, and the US Food and Drug Administration licensed an intradermal influenza vaccine in 2011. There is evidence that the dermis can be more potent than other routes of immunization. For example, in influenza vaccine trials 3 to 6 μg of hemagglutinin administered intradermally resulted in comparable immunologic responses to 15 μg administered intramuscularly.^{23,24} Furthermore, intradermal and subcutaneous injection with recombinant hepatitis B vaccine (20 μg every 2 weeks) has been compared in patients undergoing hemodialysis. The intradermal route was associated with higher rates of serologic responses and 8-fold higher antibody titers.²⁵ Similar findings have also been reported in a guinea pig model of intradermal versus subcutaneous immunization.²⁶ Our study involved much lower antigen doses. Furthermore, our data refer to therapeutic vaccination in patients with established allergic disease rather than prophylactic vaccination for prevention of infection in naive subjects.

Effective immunotherapy is associated with induction of IL-10-producing regulatory T cells²⁷⁻²⁹ and populations of forkhead box protein 3-positive T cells,^{30,31} which are also presumed regulatory T cells. These cells might directly inhibit activation of $\text{T}_\text{H}2$ cells and induce B cells to produce allergen-specific IgA and IgG isotypes, including IgG₁ and IgG₄.^{32,33} Allergen-specific non-IgE isotypes induced by means of immunotherapy exert inhibitory activity against allergen-IgE binding that can be demonstrated in functional IgE-dependent assays.¹⁵ In the present study longitudinal increases in grass pollen-specific IgG (ie, all isotypes) levels were only observed in the group receiving repeated intradermal injections of grass pollen (ie, group A), irrespective of whether the change was related to baseline values in absolute or relative terms. Relative, but not absolute, levels of allergen-specific IgG₁ also increased in group A over this period (mean 2.4-fold increase relative to baseline). These changes, although modest in comparison with those after standard subcutaneous therapy, where increases in IgG₁ and IgG₄ titers might be on the order of 20- to 40-fold,⁷ are more comparable with responses associated with sublingual immunotherapy.¹¹ However, the increase in inhibitory bioactivity of sera observed in group A in the FAB assay supports the conclusion that these changes might be of functional significance. A caveat to this conclusion is that cross-sectional comparison of increases in IgG levels and FAB inhibitory activity in group A with those seen in the control groups B and C were not statistically significant. However, as expected for such a human

population sample, a wide heterogeneity of IgG levels was observed, and the present study was not powered to detect such cross-sectional differences. Therefore definitive validation of these serologic responses will require cross-sectional comparison of clinical groups in a larger controlled trial.

The mechanism behind the effect we describe is clearly of great interest. It is possible that free allergen is transported directly through the lymphatics to regional lymph nodes for processing by dendritic cells (DCs). In an animal study radiotracer clearance from the skin to the lymph nodes was between 10- and 100-fold more rapid after intradermal than subcutaneous injection,³⁴ a finding that is consistent with imaging studies in human subjects.³⁵ Therefore intradermal allergen injection might represent a means for efficient antigen pulsing of lymph node DCs analogous to intralymphatic grass pollen immunotherapy administered as 3 injections.³⁶ However, although intralymphatic immunotherapy is unquestionably low dose (cumulative dose 3000 SQ-U) compared with subcutaneous treatment, the cumulative allergen administered in the 6 intradermal injections of our study was still 15-fold less (ie, 200 SQ-U, which is equivalent to 60 BU in total).

Inflammation and antigen exposure in the skin is also associated with maturation and trafficking of Langerhans and dermal DC populations through afferent lymphatics to regional lymph nodes, where they regulate and participate in the development of adaptive immunity or tolerance. Although several different dermal DC subsets have been described, the contribution of these subsets, or indeed epidermal Langerhans cells, to generation of antigen-specific tolerance is unknown.^{37,38} However, the relative abundance of DCs within the dermis and epidermis could also account for the potency of intradermal allergen injections in comparison with conventional subcutaneous immunotherapy injections, which target a compartment consisting mostly of connective and adipose tissue but few DCs.³⁸ DCs can express high levels of the high-affinity IgE receptor Fc ϵ RI, which facilitates allergen uptake and processing at low concentrations.³⁹ This mechanism therefore has the potential to amplify the potency of allergen given intradermally. The phenomenon we describe might also represent a similar but more direct approach to dermal DCs than that provided by means of epicutaneous delivery of allergen in patches after epithelial barrier disruption with adhesive tape stripping.⁴⁰ The presence of lower seasonal symptoms, as determined by means of global assessment, in the patients who received allergen patches provides additional support for the concept that allergen delivery superficially into the skin might be tolerogenic.

Our data have various clinical implications. First, they provide proof of concept for repeated low-dose intradermal allergen as a potential therapeutic strategy, although appropriate clinical trials are clearly required to establish whether attenuation of late responses is further associated with symptomatic improvement, as seen with existing conventional forms of immunotherapy. The need for such small quantities of allergen would make such an approach attractive.

Second, the intradermal injection given as the intervention itself induces a physiologic response (ie, the skin late response) that is quantifiable, reproducible, and suppressed by treatment. Therefore this approach affords the advantage of being inherently associated with a physiologic biomarker of allergen-specific desensitization.

Third, these data highlight the limitations of using the late response as a physiologic biomarker in clinical immunotherapy studies in which repeat measurements are being made over time.

Fourth, these data identify a novel experimental model for studying allergen-specific tolerance over a relatively short time-frame that is potentially applicable to testing novel allergens and immunomodulatory strategies, such as adjuvants.

Finally, these data might provide a rationale for apparently unrelated clinical observations, such as acquired tolerance to mosquito bites after recurrent exposures.^{41,42}

In conclusion, these data indicate a new potential strategy for inducing antigen-specific tolerance. Further studies will be required to address the underlying mechanisms and clinical utility of this approach.

We thank Dr Moises Calderon for his assistance in gaining ethical approval for this work and Dr Guy Scadding for his help with the blinding of the study.

Clinical implications: Low-dose intradermal allergen vaccination suppresses allergen-induced cutaneous late responses and induces IgG antibodies. The clinical efficacy of this approach in treating allergic disease is yet to be established.

REFERENCES

- Noon L. Prophylactic inoculation against hay fever. *Lancet* 1911;1:1572-3.
- Frankland AW, Augustin R. Prophylaxis of summer hay-fever and asthma—a controlled trial comparing crude grass-pollen extracts with the isolated main protein component. *Lancet* 1954;1:1055-7.
- Bousquet J, Lockey R, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases—a WHO position paper. *J Allergy Clin Immunol* 1998;102:558-62.
- Rinkel HJ. The management of clinical allergy. 3. Inhalant allergy therapy. *Arch Otolaryngol* 1963;77:205-25.
- Van Metre TE, Adkinson NF, Lichtenstein LM, Mardiney MR, Norman PS, 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.
- Phillips EW. Relief of hay-fever by intradermal injections of pollen extract. *JAMA* 1926;86:182-4.
- 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.
- 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.
- Walker SM, Durham SR, Till SJ, Roberts G, Corrigan CJ, Leech SC, et al. Immunotherapy for allergic rhinitis. *Clin Exp Allergy* 2011;41:1177-200.
- Phillips EW. Intradermal pollen therapy during the attack. *J Allergy* 1933;5:29-36.
- Lima MT, Wilson D, Pitkin L, Roberts A, Nouri-Aria K, Jacobson M, et al. Grass pollen sublingual immunotherapy for seasonal rhinoconjunctivitis: a randomized controlled trial. *Clin Exp Allergy* 2002;32:507-14.
- Kay AB, Ying S, Varney V, Gaga M, Durham SR, Moqbel R, et al. Messenger-RNA expression of the cytokine gene-cluster, interleukin-3 (IL-3), IL-4, IL-5, and granulocyte macrophage colony-stimulating factor, in allergen-induced late-phase cutaneous reactions in atopic subjects. *J Exp Med* 1991;173:775-8.
- Varney VA, Hamid QA, Gaga M, Ying S, Jacobson M, Frew AJ, et al. Influence of grass-pollen immunotherapy on cellular infiltration and cytokine messenger-RNA expression during allergen-induced late-phase cutaneous responses. *J Clin Invest* 1993;92:644-51.
- 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.
- 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.
- Fling JA, Ruff ME, Parker WA, Whisman BA, Martin ME, Moss RB, et al. Suppression of the late cutaneous response by immunotherapy. *J Allergy Clin Immunol* 1989;83:101-9.
- Nish WA, Charlesworth EN, Davis TL, Whisman BA, Valtier S, Charlesworth MG, et al. The effect of immunotherapy on the cutaneous late-phase response to antigen. *J Allergy Clin Immunol* 1994;93:484-93.
- Nasser SMS, Ying S, Meng Q, Kay AB, Ewan PW. Interleukin-10 levels increase in cutaneous biopsies of patients undergoing wasp venom immunotherapy. *Eur J Immunol* 2001;31:3704-13.
- Meiler F, Zunkler J, Klunker S, Ruckert B, Akdis CA, Akdis M. In vivo switch to IL-10-secreting T regulatory cells in high dose allergen exposure. *J Exp Med* 2008;205:2887-98.
- Krishna MT, Ewan PW, Diwakar L, Durham SR, Frew AJ, Leech SC, et al. Diagnosis and management of hymenoptera venom allergy: British Society for Allergy and Clinical Immunology (BSACI) guidelines. *Clin Exp Allergy* 2011;41:1201-20.
- Muller UR, Dudler T, Schneider T, Cramer R, Fischer H, Skrbic D, et al. Type I skin reactivity to native and recombinant phospholipase A2 from honeybee venom is similar. *J Allergy Clin Immunol* 1995;96:395-402.
- Worm M, Lee HH, Kleine-Tebbe J, Hafner RP, Laidler P, Healey D, et al. Development and preliminary clinical evaluation of a peptide immunotherapy vaccine for cat allergy. *J Allergy Clin Immunol* 2011;127:89-97.
- Belshe RB, Newman FK, Cannon J, Duane C, Treanor J, Van Hoecke C, et al. Serum antibody responses after intradermal vaccination against influenza. *N Engl J Med* 2004;351:2286-94.
- Kenney RT, Frech SA, Muenz LR, Villar CP, Glenn GM. Dose sparing with intradermal injection of influenza vaccine. *N Engl J Med* 2004;351:2295-301.
- Propst T, Propst A, Lhotka K, Vogel W, König P. Reinforced intradermal hepatitis B vaccination in hemodialysis patients is superior in antibody response to intramuscular or subcutaneous vaccination. *Am J Kidney Dis* 1998;32:1041-5.
- Matriano JA, Cormier M, Johnson J, Young WA, Buttery M, Nyam K, et al. Macroflux microprojection array patch technology: a new and efficient approach for intracutaneous immunization. *Pharm Res* 2002;19:63-70.
- 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.
- 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.
- Jutel M, Akdis M, Budak F, Aebischer-Casaulta C, Wrzyszczyk 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.
- 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, e1.
- Aslam A, Chan H, Warrell DA, Misbah S, Ogg GS. Tracking antigen-specific t-cells during clinical tolerance induction in humans. *PLoS One* 2010;5:e11028.
- 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.
- Satoguina JS, Adjibimey T, Arndts K, Hoch J, Oldenburg J, Layland LE, 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.
- Kersey TW, Van Eyk J, Lannin DR, Chua AN, Tafta L. Comparison of intradermal and subcutaneous injections in lymphatic mapping. *J Surg Res* 2001;96:255-9.
- O'Mahony S, Solanki CK, Barber RW, Mortimer PS, Purushotham AD, Peters AM. Imaging of lymphatic vessels in breast cancer-related lymphedema: intradermal versus subcutaneous injection of 99mTc-immunoglobulin. *AJR Am J Roentgenol* 2006;186:1349-55.
- Senti G, Prinz Vavricka BM, Erdmann I, Diaz MI, Markus R, McCormack SJ, et al. Intralymphatic allergen administration renders specific immunotherapy faster and safer: a randomized controlled trial. *Proc Natl Acad Sci U S A* 2008;105:17908-12.
- Novak N, Bieber T. 2. Dendritic cells as regulators of immunity and tolerance. *J Allergy Clin Immunol* 2008;121(suppl):S370-4.
- Romani N, Flacher V, Tripp CH, Sparber F, Ebner S, Stoitzner P. Targeting skin dendritic cells to improve intradermal vaccination. *Curr Top Microbiol Immunol* 2012;351:113-38.
- Maurer D, Fiebigler S, Ebner C, Reininger B, Fischer GF, Wichlas S, et al. Peripheral blood dendritic cells express Fc epsilon RI as a complex composed of Fc epsilon RI alpha- and Fc epsilon RI gamma-chains and can use this receptor for IgE-mediated allergen presentation. *J Immunol* 1996;157:607-16.
- Senti G, Graf N, Haug S, Rüedi N, von Moos S, Sonderegger T, et al. Epicutaneous allergen administration as a novel method of allergen-specific immunotherapy. *J Allergy Clin Immunol* 2009;124:997-1002.
- Reunala T, Brummerkorvenkontio H, Palosuo T. Are we really allergic to mosquito bites. *Ann Med* 1994;26:301-6.
- Peng Z, Ho MK, Li C, Simons FE. Evidence for natural desensitization to mosquito salivary allergens: mosquito saliva specific IgE and IgG levels in children. *Ann Allergy Asthma Immunol* 2004;93:553-6.

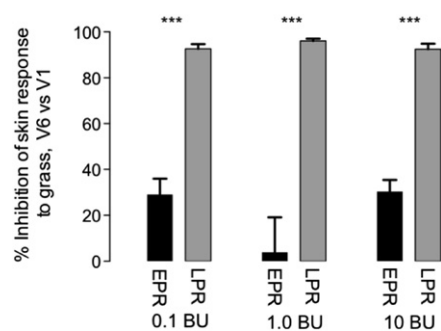


FIG E1. Comparison of inhibition of early versus late responses after repeated intradermal grass pollen injections in group A. Data shown represent the mean $\pm$ SEM percentage inhibition of early- and late-response area at visit 6 compared with visit 1. Significance was assessed with the paired Student *t* test. ****P* < .001. *EPR*, Early-phase response; *LPR*, late-phase response.