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Short ragweeds is highly cross-reactive with other ragweeds

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ABSTRACT

Background: The most widespread ragweed (*Ambrosia*) species in North America are short ragweed (*Ambrosia artemisiifolia*; Amb a), giant ragweed (*Ambrosia trifida*; Amb t), and western ragweed (*Ambrosia psilostachya*; Amb p). Varied geographic distributions of ragweed species raise questions regarding the need for ragweed species-specific allergen immunotherapy.

Objective: To determine allergenic cross-reactivity among ragweed species by immunologic analyses of sera from subjects allergic to ragweed from North America and Europe.

Methods: Sera were collected from 452 subjects allergic to ragweed who participated in Amb a sublingual immunotherapy tablet clinical trials. All subjects had positive skin prick test and serum IgE against Amb a. Ragweed-specific IgE (pre treatment) and IgG4 (post treatment) were measured by ImmunoCAP. IgE inhibition studies among Amb a, Amb t, and Amb p were conducted. Using pooled sera from another ragweed-allergic population, IgE inhibition studies of 7 less widespread *Ambrosia* species also were conducted.

Results: A strong correlation between Amb a vs Amb p and Amb t serum IgE levels was observed. In the vast majority of pretreatment sera, Amb a inhibited Amb a, Amb p, and Amb t IgE reactivity by more than 90%. Strong correlations were observed between Amb a vs Amb p and Amb t post-treatment IgG4 levels. In pooled sera, Amb a extract inhibited the binding of serum IgE to all 10 ragweed species by 98%–100%.

Conclusion: In a population of subjects allergic to Amb a, substantial allergenic cross-reactivity among Amb a, Amb p, and Amb t was demonstrated, indicating Amb a–based single-species ragweed allergen immunotherapy is clinically sufficient for patients exposed to diverse ragweed pollens.

Trial Registry: Clinicaltrials.gov, NCT00770315, NCT00783198, and NCT00330083.

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Introduction

Ragweed allergic rhinitis with or without conjunctivitis (AR/C) is common in the United States, with an estimated prevalence of ragweed sensitization of approximately 26%.¹ The cause of ragweed allergy is IgE sensitization and exposure to pollen from plants of the genus *Ambrosia*, which in North America comprises more than 50 species (<http://www.theplantlist.org/browse/A/Compositae/Ambrosia/>), of which short ragweed (*Ambrosia artemisiifolia*;

Amb a), giant ragweed (*Ambrosia trifida*; Amb t), and western ragweed (*Ambrosia psilostachya*; Amb p) are the most widespread; Amb a is the most ubiquitous species.² Although the 3 main ragweed species are generally distributed across North America, colocalization is not complete, which could affect local pollen exposure and raise questions regarding the need for ragweed species-specific diagnosis and treatment.

Allergy immunotherapy (AIT) is recognized as a well-tolerated and efficacious means of not only alleviating AR/C symptoms but also altering the underlying disease and providing sustained clinical relief after completion of treatment.^{3,4} In recent years, substantial evidence for the clinical efficacy and safety of sublingual allergy immunotherapy (SLIT) has been published. A number of clinical trials have led to the approval of 2 grass pollen SLIT tablets in Europe and North America. In addition, 2 pivotal double-blinded, placebo-controlled multicenter clinical trials have led to the recent approval of a ragweed SLIT tablet product in the United States and Canada.^{5,6}

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Extensive allergenic cross-reactivity among different ragweed species has been described,^{7,8} and the possibility of diagnosis and treatment of ragweed allergy with a single ragweed species has been proposed with reference to allergenic cross-reactivity.⁹ Accordingly, current US immunotherapy guidelines propose that when allergens are substantially cross-reactive, the selection of a single pollen within that genus or subfamily might suffice.¹⁰ The same argumentation is found in European guidelines that state efficacy evaluations of related and cross-reactive allergenic species assigned within a “homologous group” may be extrapolated to other species in the same group.¹¹ Thus, if substantial cross-reactivity can be established within one homologous group, then it supports the efficacy of AIT with one representative allergen.

This article reports on post hoc immunologic analyses of sera from subjects allergic to ragweed in North America and Europe with positive skin prick test (SPT) and serum IgE against Amb a who participated in the Amb a ragweed SLIT tablet clinical trials.^{5,6} The 2 trials were conducted in 80 centers across the United States and Canada,⁶ 84 centers in the United States and Canada, and 30 centers in Eastern Europe.⁵ The hypothesis of this analysis was that, owing to substantial allergenic cross-reactivity among different ragweed species, treatment with the Amb a ragweed SLIT tablet would induce strongly cross-reacting IgG4 responses against Amb a, Amb p, and Amb t, indicating clinical relevance of the single-species ragweed SLIT tablet against a broader range of pollen exposure to ragweed species. IgE inhibition studies among Amb a, Amb t, and Amb p and 7 additional less widespread *Ambrosia* species also were conducted using pooled sera from subjects allergic to ragweed in the United States.

Methods

Design of Trials Used for Individual Sera Analyses

Individual sera for this analysis were collected from subjects who participated in 2 pivotal clinical trials of the standardized short ragweed SLIT tablet (1 unit of Amb a major allergen [Amb a 1 U]; MK-3641; Merck & Co, Inc, Kenilworth, New Jersey; ALK, Hørsholm, Denmark); the details and results have been previously described.^{5,6} Briefly, these 2 trials were randomized, double-blinded, placebo-controlled trials in which subjects received a daily ragweed SLIT tablet or placebo for 52 weeks. Only subjects receiving the approved US Food and Drug Administration dose of 12 Amb a 1 U or placebo were included in this analysis.

Subjects in the 2 trials were 18 to 50 years old with a physician-diagnosed history of ragweed-induced AR/C with or without asthma. Key inclusion criteria were a positive SPT reaction to Amb a (ALK) at screening and a positive specific IgE reaction against Amb a (IgE class at least 2; ≥ 0.7 kU/L) measured by ImmunoCAP (Thermo Fischer Scientific, Uppsala, Sweden).

The protocol for the 2 trials was approved by the institutional review boards, and written consent was obtained from all subjects. The studies are registered at clinicaltrials.gov (identifiers NCT00770315 and NCT00783198).

Analyses of Individual Sera

ImmunoCAP IgE and IgG4 correlation studies

For correlation studies, serum specific IgE (sIgE) and specific IgG4 (sIgG4) to Amb a, Amb p, and Amb t were measured by standard ImmunoCAP (Phadia 250; Thermo Fischer Scientific). Specific IgE was measured in pretreatment sera and sIgG4 was measured in post-treatment sera.

ImmunoCAP IgE inhibition study

For the ImmunoCAP IgE inhibition study, an experimental inhibitor extract of Amb a was made: 15 g of defatted pollen collected in the United States was extracted in 150 mL of NH_4HCO_3 (0.125

mol/L, pH 8.3) at 5°C for 3 hours followed by dialysis (MWCO 3.5K), filtration (0.2 μm), and freeze drying. Sera were preincubated overnight at 4°C with 3 mg/mL of Amb a extract or rHSA (Novozymes, Bagsværd, Denmark) as a negative control. The following day, the extent of residual IgE binding in individual sera against Amb a, Amb p, and Amb t was measured using standard tests on a Phadia 250 system (Thermo Fischer Scientific). The number of samples in each group shown in the corresponding results reflects that subjects' sera were included only if sIgE reaction of the non-inhibited sample was at least 0.7 kU/L toward Amb a, Amb p, or Amb t, respectively.

Inclusion Criteria for Pooled Sera Analysis

Sera from 29 adult subjects enrolled in a phase 1 trial of a ragweed SLIT tablet (RT-01; www.clinicaltrials.gov, identifier NCT00330083)¹² were collected before and after treatment to generate a serum pool from ragweed-sensitized subjects. The trial was conducted at 2 centers in the United States (San Antonio, Texas, and Normal, Illinois). Only sera from subjects with a positive SPT reaction to Amb a, sIgE reaction to Amb a 1 of at least class 2 (corresponding to ≥ 0.7 kU/L), and sIgE reaction to profilin, horseradish peroxidase, and *Artemisia vulgaris* lower than class 2 were included in the serum pool. The serum pool was compiled of pre-treatment sera from subjects in the ragweed SLIT tablet group and post-treatment sera from subjects in the placebo group.

Analysis of Pooled Sera

To prepare the pollen extracts used in the pooled sera analysis, pollens from 10 commercially available ragweed species were obtained from Biopol Laboratory, Inc (Spokane, Washington):

Amb a: *Ambrosia artemisiifolia* (short or common ragweed), catalog number WP8014

Amb t: *Ambrosia trifida* (giant ragweed), catalog number WP8016

Amb p: *Ambrosia psilostachya* (western ragweed), catalog number WP8015

Amb dum: *Ambrosia dumosa* (desert ragweed), catalog number WP8013

Amb del: *Ambrosia deltoidea* (rabbit bush), catalog number WP8012

Amb con: *Ambrosia confertiflora* (slender ragweed), catalog number WP8011

Amb bip: *Ambrosia bipinnatifida* (beach sandbur), catalog number WP8010

Amb bid: *Ambrosia bidentata* (southern ragweed), catalog number WP8009

Amb amb: *Ambrosia ambrosioides* (canyon ragweed), catalog number WP8008

Amb ac: *Ambrosia acanthicarpa* (false ragweed or annual bursage), catalog number WP8007

Pollen from each species (30 g) was extracted in 300 mL of NH_4HCO_3 (0.125 mol/L, pH 8.3) at 5°C for 2 hours followed by dialysis (MWCO 3.5K), filtration (0.2 μm), and freeze drying. A 500-mg aliquot of each lyophilized extract was subsequently gel-filtered using a HiLoad 16/60 Superdex 75 column connected to an ÄKTA explorer 100 Air system (GE Healthcare, Chalfont, United Kingdom).

For the IgE inhibition study of the pooled sera, an aliquot of each of the 10 ragweed extracts was biotinylated for use within the ADVIA Centaur (Siemens Medical Solutions, Diagnostics, Tarrytown, New York) inhibition assay together with non-labeled inhibitor extracts (Amb a, Amb p, and Amb t). Briefly, the sIgE level of the ragweed serum pool was determined against the 10 biotin-labeled ragweed extracts before and after the addition of 1 mg/mL of non-labeled inhibitor extracts (Amb a, Amb p, and Amb t, respectively), similar to a previously described methodology.¹³

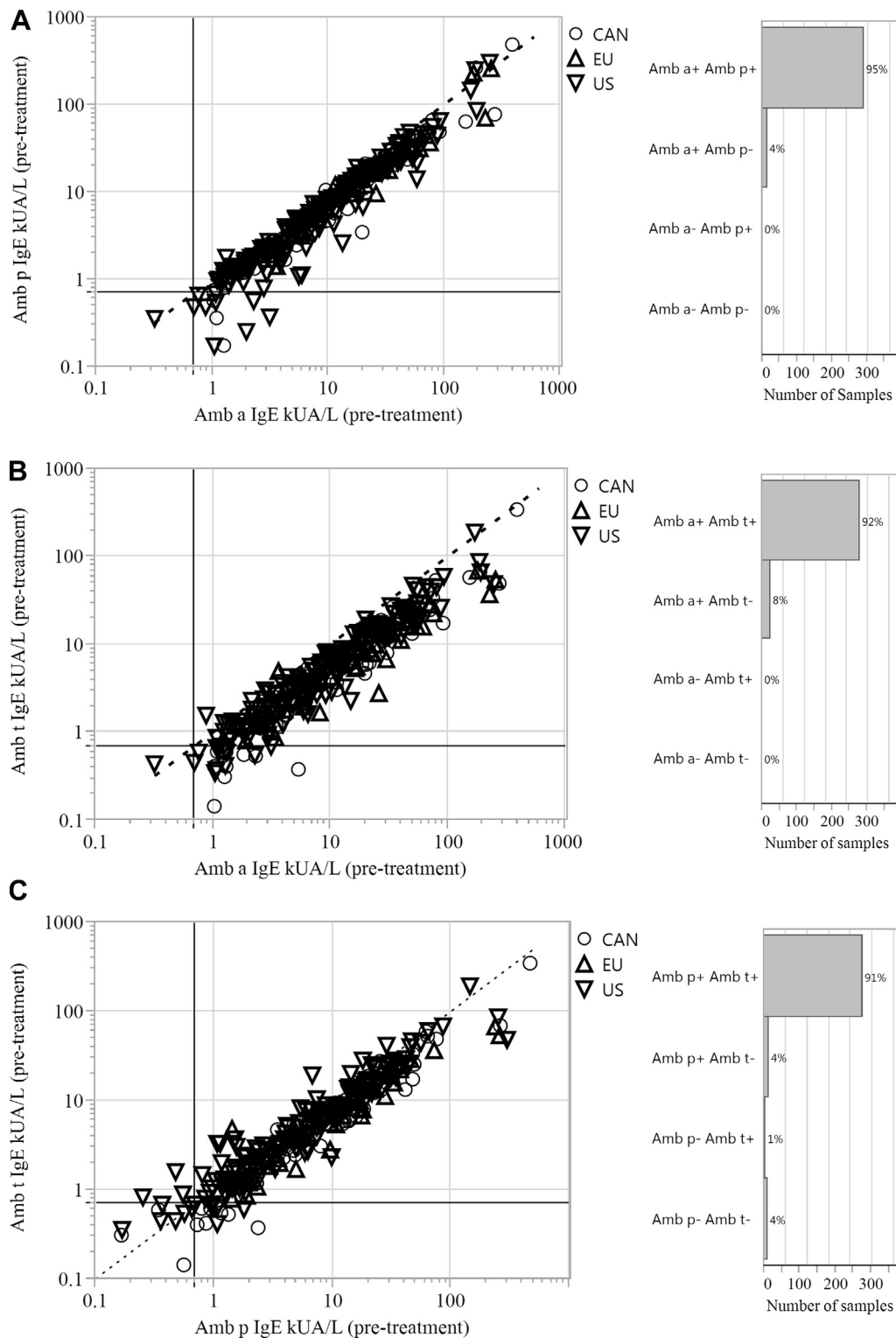


Figure 1. Correlation between IgE reactivity for (A) Amb a vs Amb p, (B) Amb a vs Amb t, and (C) Amb p vs Amb t. The dotted diagonal line indicates the line of identity (slope = 1, intercept = 0) representing ideal correlation, and the solid vertical and horizontal lines indicate 0.7 kU/L of IgE (trial inclusion criterion level [Amb a]). The correlation coefficients for the pretreatment association between $\log_{10}$ Amb a IgE vs $\log_{10}$ Amb p IgE, $\log_{10}$ Amb a IgE vs $\log_{10}$ Amb t IgE, and $\log_{10}$ Amb p IgE vs $\log_{10}$ Amb t IgE were 0.9644, 0.9532, and 0.9505, respectively. Amb a, *Ambrosia artemisiifolia*; Amb t, *Ambrosia trifida*; Amb p, *Ambrosia psilostachya*; CAN, Canada; EU, European Union; US, United States.

Statistical Analysis

All statistical calculations were carried out using SAS JMP 11.0.0 (SAS Institute, Cary, North Carolina). *P* values less than .05 were considered significant. According to the Steel-Dwass nonparametric method, there was a significant difference between the

change in the level of sIgG4 ($\Delta[\text{sIgG4}] = [\text{post-treatment value} - \text{pretreatment value}]$) for all tested species (Amb a, Amb p, and Amb t) in the US and Canadian geographic regions when comparing the placebo and 12 Amb a 1 U groups. Also, according to the Steel-Dwass nonparametric method, there was no significant difference

between Δ slgG4 levels determined for the different regions and treatment group, irrespective of the species (Amb a or Amb p or Amb t) used to measure slgG4 level.

Appropriate nonparametric statistical tests were used whenever non-normality or variance in homogeneity was observed.

Results

Demographics and Geographic Distribution of Trial Subjects

Demographics and background characteristics of the subjects enrolled into the 2 trials have been published.^{5,6} Serum samples were available from a total of 452 subjects who received the ragweed SLIT tablet 12 Amb a 1 U or placebo. The number of subjects by region in each study is presented in detail in eTable 1.

Correlation of Pretreatment Ragweed IgE Levels

Serum Amb p and Amb t IgE was quantified and correlated with the corresponding slgE level of Amb a. Figure 1 shows a high correlation between subject serum slgE levels of Amb a vs Amb p and Amb t, respectively. Most sera were positive (slgE ≥ 0.7 kU/L) for the 2 species ($>90\%$ concordance). The correlations between slgE levels to Amb a vs Amb p and Amb t, respectively, trended slightly toward higher Amb a slgE levels, which could be an effect of selecting sera based on Amb a reactivity (Fig 1A, B). This is supported by the almost complete correlation observed between Amb p and Amb t slgE levels (Fig 1C). The same relative magnitude of Amb a, Amb p, and Amb t IgE reactivity was seen in sera collected from the United States, Canada, and Europe, respectively, with no significant differences observed.

IgE Inhibition Studies

Figure 2 shows that pre-incubation of sera with Amb a extract inhibited Amb p and Amb t IgE reactivity by greater than 90% in 98% of subjects (same level as the positive control, ie, Amb a inhibition of Amb a-specific IgE reactivity), indicating a high degree of overlap and similarity of IgE epitopes among the 3 ragweed species.

Correlation of Treatment-Induced Ragweed IgG4

The correlations between Amb a vs Amb p and Amb t post-treatment slgG4 levels were almost identical to the pattern seen for pretreatment IgE, with a high correlation between slgG4 levels of the different species and a slight trend toward higher Amb a slgG4 levels (Fig 3). This result supports that the strong correlation seen for pretreatment slgE is replicated by the slgG4 response induced by the Amb a SLIT tablet. No statistically significant differences in induced slgG4 levels were observed among sera collected from the 3 regions (United States, Canada, and Europe).

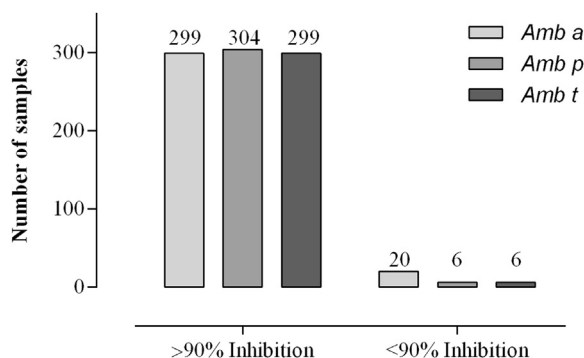


Figure 2. Number of individual subjects' sera (pretreatment) whose IgE reactivity toward Amb a, Amb p, and Amb t, respectively, was inhibited more than 90% (left) or less than 90% (right) by pre-incubation with Amb a extract. Amb a, *Ambrosia artemisiifolia*; Amb t, *Ambrosia trifida*; Amb p, *Ambrosia psilostachya*.

Cross-Reactivity with Other Ragweed Species

In addition to the 3 major ragweed species Amb a, Amb p, and Amb t, other less ubiquitously distributed ragweed species are found across the United States, 7 of which were assessed for cross-reactivity using pooled sera from a ragweed-sensitive population ($N = 29$). Unlabeled extracts of Amb a, Amb p, or Amb t inhibited IgE reactivity by 60% to 100% against a panel of 10 ragweed species. Although Amb p was the overall least effective inhibitor, followed by Amb t, the Amb a extract inhibited the binding of serum slgE to all 10 ragweed species by 98% to 100% (Fig 4). This demonstrates that ragweed slgE in sera from subjects diagnosed with short ragweed allergy is strongly cross-reactive with ragweed allergens from major and minor ragweed species.

Discussion

Ragweed is ubiquitous across North America, with the most widespread species being short ragweed (Amb a), western ragweed (Amb p), and giant ragweed (Amb t). However, several other species exist that might be potential targets for AIT. The subjects included in the first part of this analysis participated in 2 trials with the objective of determining the clinical efficacy of an Amb a-based SLIT tablet. Subjects were included based on clinical history of ragweed allergy, positive SPT reaction to Amb a, and presence of Amb a serum slgE. Furthermore, subjects were recruited from large regions of North America (sites were located mostly east of the Rocky Mountains, where multiple ragweed species are prevalent) and from Europe, where Amb a is the dominating ragweed species. In this population, the correlations of pretreatment serum slgE and treatment-induced slgG4 toward extracts of the 3 main ragweed species in the United States, respectively, were significant. Despite the preselection of subjects allergic to Amb a, there was a very strong pairwise correlation between the quantitative binding of IgE and IgG4 to Amb a vs Amb p and Amb t, respectively. Correlation of subjects' IgE reactivity toward extracts of related ragweed species is a strong indicator of allergenic cross-reactivity, but it does not discriminate between true cross-reactivity on the IgE epitope level and co-sensitization. Direct evidence for true IgE epitope cross-reactivity among the 3 ragweed species was obtained by inhibition experiments in which extract from Amb a abolished binding of subject slgE to Amb p and Amb t. In the second part of this study, Amb a almost completely inhibited binding to pooled ragweed-sensitive sera by extracts of 7 minor *Ambrosia* species.

These experiments showed that the Amb a-reactive antibody epitope repertoire was dominant; in individual clinical trial sera and in studies with pooled ragweed allergic sera, the Amb a extract competed very efficiently with extract from all other ragweed species for the reactivity of serum slgE. Although the high level of cross-reactivity among ragweed species indicated otherwise, different results might have been obtained with a different preselection of subjects. However, if this is true, then it would serve to emphasize that allergenic cross-reactivity in a clinical context is valid only for a patient population selected with the same inclusion criteria as in the original study. Furthermore, it would have been of interest to break down the analysis into smaller geographic regions where certain ragweed species are more dominant than others. However, the sample size for smaller regions was not adequate to perform a meaningful analysis.

The clinical relevance of allergenic cross-reactivity in AIT has been acknowledged by authorities in the United States and in Europe.^{10,11,14} US guidelines suggest that AIT regimens of only a single species that is "substantially cross-reactive" to other related species could suffice.¹⁰ The precedence for clinically efficacious immunotherapy with a single species that is representative of a larger group of related species comes from grass pollen allergy; the clinical

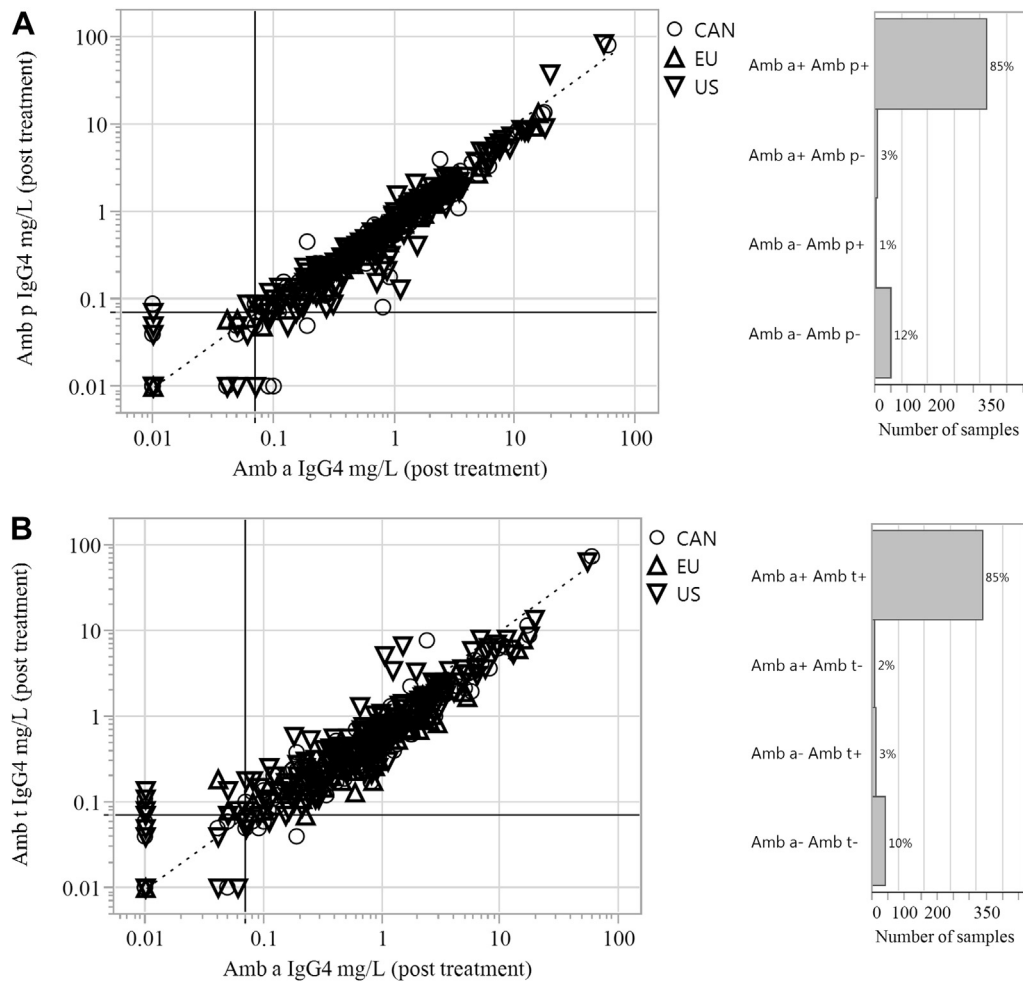


Figure 3. Correlation between post-treatment specific IgG4 for (A) Amb a vs Amb p and (B) Amb a vs Amb t. The dotted diagonal line indicates the line of identity (slope = 1, intercept = 0) representing ideal correlation, and the solid vertical and horizontal lines denote the lower limit of quantitation for the assay (0.07 mg/L). The correlation coefficients for the post-treatment association among $\log_{10}$ Amb a IgG4 vs $\log_{10}$ Amb p IgG4 and $\log_{10}$ Amb a IgG4 vs $\log_{10}$ Amb t IgG4 were 0.9697 and 0.9408, respectively. Amb a, *Ambrosia artemisiifolia*; Amb t, *Ambrosia trifida*; Amb p, *Ambrosia psilostachya*; CAN, Canada; EU, European Union; US, United States.

efficacy of a SLIT tablet based on the representative grass species *Phleum pratense* has been demonstrated by several large clinical trials in Europe and the United States.^{3,15–17} A practical demonstration of clinically relevant allergenic cross-reactivity among ragweed

species was provided by Jacobs et al¹⁸ who demonstrated that ragweed-sensitized patients exposed to Amb a pollen in an environmental exposure challenge chamber had AR/C symptoms that mimicked those elicited by Amb t during the natural season.

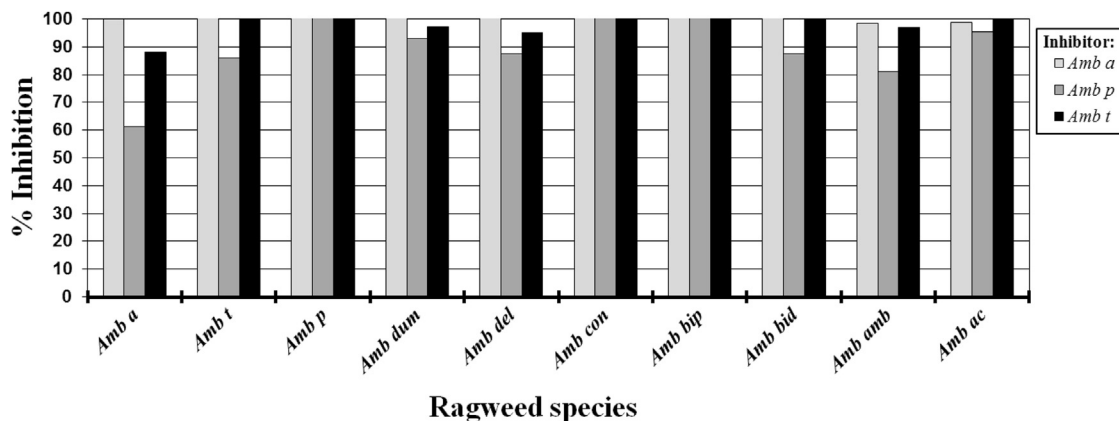


Figure 4. IgE cross-reactivity among 10 ragweed species. IgE cross-reactivity among the 3 major ragweed species (Amb a, Amb p, and Amb t) and 7 minor ragweed species in the United States was assessed by ADVIA Centaur inhibition using a serum pool composed of single sera collected from 29 subjects allergic to ragweed in the US. Amb a, *Ambrosia artemisiifolia*; Amb ac, *Ambrosia acanthicarpa*; Amb amb, *Ambrosia ambrosioides*; Amb bid, *Ambrosia bidentate*; Amb bip, *Ambrosia bipinnatifida*; Amb dum, *Ambrosia dumosa*; Amb con, *Ambrosia confertiflora*; Amb del, *Ambrosia deltoidea*; Amb p, *Ambrosia psilostachya*; Amb t, *Ambrosia trifida*.

A small European study reported by Asero et al.¹⁹ described a certain level of heterogeneity in a population of 41 patients from an area dominated by Amb a. Of the 41 patients, 35 had positive SPT reactions for Amb a and Amb t and the remaining 6 showed a positive SPT reaction only for Amb a. Because it is most likely that in this particular population the sensitizing species was Amb a, the finding that 85% of the population exhibited a positive SPT reaction to Amb t indicates a large degree of allergenic cross-reactivity between the 2 species. As noted by the investigators, the finding that 15% of the population had a positive SPT reaction only to Amb a further indicates that, in addition to allergenic cross-reactivity, allergenic differences exist between the 2 species. This is not surprising because related, but distinct, species by definition are not identical. Immunologic cross-reactivity can be examined at many levels, and it is well established that a higher-resolution assays are able to detect more specific immune responses. The authors and others have demonstrated that monoclonal responses toward allergens, be they antibody²⁰ or T-cell²¹ responses that include individual epitopes, can differentiate between allergen homologues from different species or even individual allergen isoforms from the same species. These findings reflect a key characteristic of the vertebrate immune system, namely diversity obtained through the selection of highly complex polyclonal repertoires. Therefore, it is highly likely that the immunologic cross-reactivity for the various related, but nonidentical, *Ambrosia* species demonstrated in the present study consists of the combined effects of individual antibody clones with various degrees of cross-reactivity that can range from species-specific to fully cross-reactive.²² However, the characteristics of individual clones are not indicative of the combined activity of a subjects' polyclonal immune response. The fact that Asero et al.¹⁹ identified a subpopulation that reacted only to Amb a when using a high-level assay such as SPT could be due to differences in the patients' sensitization patterns because some individual *Ambrosia* allergens are known to be more immunologically cross-reactive than others.²³ Furthermore, unlike the United States, where ragweed species are abundant, ragweed, particularly Amb a, is a recent addition to the European flora, and in the absence of co-exposure to multiple related species, the ragweed-reactive antibody repertoires of European patients allergic to ragweed are likely to be more focused on the dominating species. The finding in this study of the same relative reactivity of pretreatment sIgE and post-treatment sIgG4 toward Amb a, Amb p, and Amb t, respectively, in European and North American subjects, emphasizes the relevance of the single-species ragweed SLIT tablet for patients with Amb a–sensitized ragweed allergy in regions with mono- and multiple-species exposure.

In conclusion, the subjects in these 2 pivotal ragweed SLIT tablet clinical trials examined in this study represent, to date, the largest population of subjects sensitized to ragweed to be examined for allergenic cross-reactivity. The results demonstrate extensive allergenic cross-reactivity among Amb a, Amb p, and Amb t in patients diagnosed with IgE toward Amb a. AIT with Amb a in this population also induced sIgG4 toward Amb p and Amb t, suggesting a meaningful immunologic response of the underlying cross-reactivity. Additional studies demonstrated extensive cross-reactivity between Amb a and 7 minor *Ambrosia* species. The data from this analysis support the use of an Amb a–based single-species ragweed AIT against related ragweed species in regions of North America and Europe with diverse ragweed pollen exposure.

Acknowledgments

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Supplementary Data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.anai.2015.09.016>.

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eTable 1

Number of subjects by region whose sera were analyzed for each ragweed species

Species	Isotype	Treatment status	Study P05233				Study P05234						Total, N
			Canada		US		Canada		US		EU		
			12 Amb a 1 U, n	Placebo, n	12 Amb a 1 U, n	Placebo, n	12 Amb a 1 U, n	Placebo, n	12 Amb a 1 U, n	Placebo, n	12 Amb a 1 U, n	Placebo, n	
Amb a	IgE	before	67	57	90	43	25	0	77	0	45	0	404
Amb p	IgE	before	67	0	90	0	25	0	77	0	45	0	304
Amb t	IgE	before	67	0	90	0	25	0	77	0	45	0	304
Amb a	IgG4	after	67	57	90	N/A	25	0	73	N/A	44	47	403
Amb p	IgG4	after	67	57	90	N/A	25	0	73	N/A	44	0	356
Amb t	IgG4	after	67	57	90	N/A	25	0	77	N/A	45	0	361
Total, N			67	57	90	43	25	0	77	0	45	47	451

Abbreviations: Amb a, *Ambrosia artemisiifolia*; Amb t, *Ambrosia trifida*; Amb p, *Ambrosia psilostachya*; EU, European Union; N/A, not available; US, United States.