

Giant ragweed specific immunotherapy is not effective in a proportion of patients sensitized to short ragweed: Analysis of the allergenic differences between short and giant ragweed

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Background: Short ragweed and giant ragweed pollen allergens are considered largely cross-reactive, and it is generally believed that 1 species is sufficient for skin testing and immunotherapy. However, in the area north of Milan (a zone widely invaded only by short ragweed), about 50% of patients submitted to injection specific immunotherapy with giant ragweed showed little or no clinical response, but showed an excellent outcome if they were shifted to short ragweed specific immunotherapy.

Objective: To investigate allergenic differences between short and giant ragweed.

Methods: IgE reactivity to short ragweed of sera from 16 patients allergic to ragweed was assessed by immunoblot before and after absorption with short and giant ragweed. Moreover, 41 ragweed-monosensitive patients underwent skin prick test with both ragweed species.

Results: In several cases, preabsorption of sera with giant ragweed extract was unable to inhibit IgE reactivity fully against both a 43-kd allergen and other allergens at different molecular weights in short ragweed. On skin prick test, short ragweed induced larger wheals than giant ragweed in the majority of patients, and 6 of 41 (15%) patients were strongly short ragweed–positive but giant ragweed–negative. The immunoblot with the serum from 1 of these subjects showed a strong IgE reactivity to short ragweed at about 43 kd in the absence of any reactivity to giant ragweed.

Conclusion: Short and giant ragweed are not allergenically equivalent. Allergenic differences involve both the major allergens Amb a 1-2/Amb t 1-2 and some minor allergens. In patients allergic to ragweed, both diagnosis *in vivo* and immunotherapy should always be performed by using the ragweed species present in that specific geographic area. (J Allergy Clin Immunol 2005;116:1036-41.)

Key words: Ragweed, allergy, cross-reactivity, specific immunotherapy, allergens

Abbreviations used

SIT: Specific immunotherapy

SPT: Skin prick test

Ragweeds, including *Ambrosia artemisiifolia* (short ragweed) and *Ambrosia trifida* (giant ragweed) have always represented one of the most important causes of seasonal respiratory allergy in the United States. These weeds are highly invasive pioneer plants and, starting from the United States, have invaded Europe and Japan.¹ In the last decade, short ragweed has also become a relevant allergen in Italy, particularly in the area north of Milan. Although the first cases of ragweed pollen allergy in this area were reported only in the late 1980s,² ragweed currently represents the second most frequent cause of respiratory allergy after grass pollen,³ affecting both young people and older subjects.⁴ In the Milan area, patients allergic to ragweed frequently have severe respiratory symptoms that are not sufficiently controlled by drug therapies alone, and they are therefore good candidates for allergen specific immunotherapy (SIT). SIT is currently the only available treatment able to modify the natural course of the allergic disease and represents a cornerstone in the treatment of airborne allergies. Its efficacy and long-term effect in reducing allergic symptoms have been demonstrated by several properly performed clinical studies and are now widely accepted.⁵⁻⁹ As a consequence, a large number of patients allergic to ragweed living in this area have undergone injection SIT with ragweed extracts at this allergy unit during the last 10 years.

In Italy, ragweed pollen extracts for skin prick test (SPT) and immunotherapy have generally been marketed under the generic name of *ragweed*; however, some contain short ragweed pollen, some giant ragweed pollen, and others a 50%-50% mixture of both species. At this allergy center, extracts from different producers have been prescribed for therapeutic use. Over time it was observed that, although all patients were treated by the same physician (R. A.) following the same perennial schedule, nearly all patients receiving short ragweed injection

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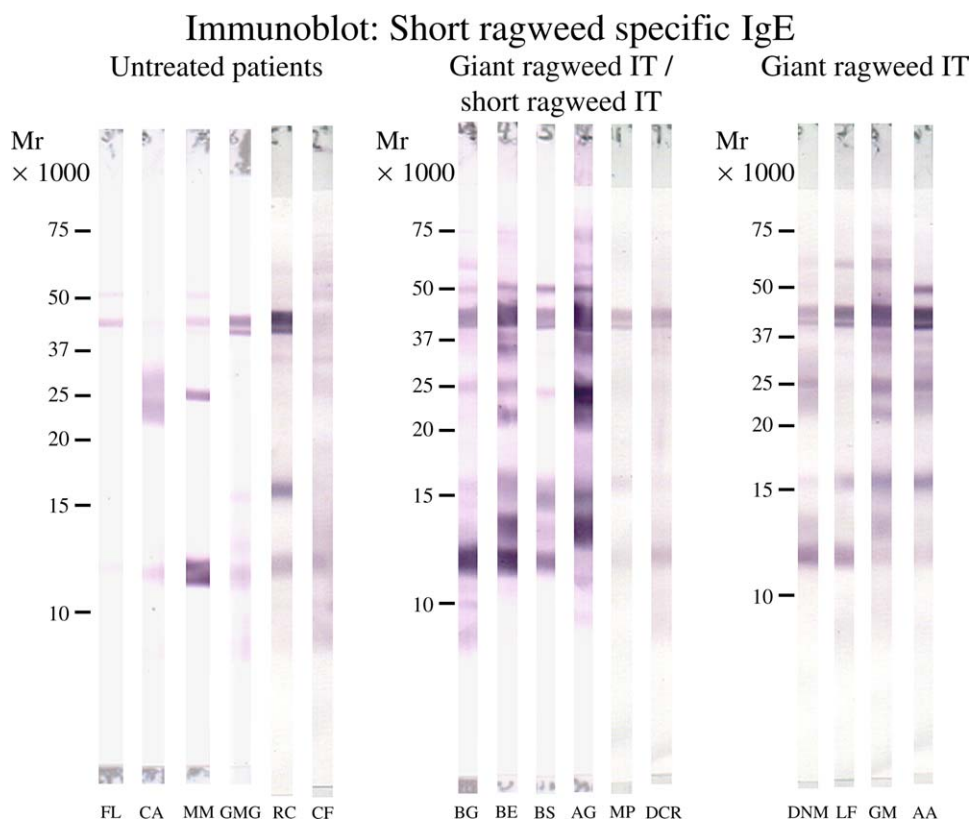


FIG 1. Short ragweed immunoblot analysis with sera from all 16 patients included in the cross-sectional part of the study. IgE reactivity to several allergens at different molecular weights can be observed, although reactivity at about 43 kd (Amb a 1) is virtually always present. No reactivity at 5 kd (Amb a 5) can be appreciated. *IT*, Immunotherapy.

immunotherapy (SIT) reported excellent results, whereas this was the case in only about 1/2 of the patients treated with giant ragweed extract, as confirmed by both symptom scores and the use of rescue therapy (either β -agonists or antihistamines). Interestingly, most patients reporting poor benefit from SIT had reached the maximum recommended doses in the absence of any local or systemic reaction, whereas most responders had intense local reactions after the injection during the build-up phase, and in several cases, patients were unable to reach the maximum scheduled dose because of the risk of systemic adverse reactions. Further, if patients nonresponding to 1 to 2 years of giant ragweed SIT at maximum dose were transferred to short ragweed SIT, they started experiencing severe local reactions at relatively low doses during the build-up phase, as if they were being treated with a totally new allergen, and they frequently had to be given very low maintenance doses because of the onset of systemic reactions. In these patients, the clinical response to short ragweed SIT was excellent. Taken together, these observations suggested possible allergenic differences between short and giant ragweed pollen causing a poor response to giant ragweed SIT in short ragweed-sensitized patients.

Early studies based on RAST inhibition studies found that cross-reactivity between ragweeds is very strong as a result of the similarity between group 1 and 2 major

allergens^{10,11}; more than 90% of patients allergic to ragweed show IgE specific for these 2 allergens.¹² However, more recent studies show that among minor allergens, group 5 allergens, namely Amb a 5 and Amb t 5, although similar in molecular weight and amino acid composition (sequence homology 46%), show little or no cross-reactivity.¹³⁻¹⁷ Because only as many as 20% of subjects allergic to ragweed in the United States appear to react to this protein,¹² it seemed rather unlikely that the high prevalence of poor responses to giant ragweed SIT could be a result of sensitization to this allergen. We tried to clarify this issue in the current study, looking for possible allergenic differences between short and giant ragweed pollen.

METHODS

Study design

On the basis of the preliminary clinical observations reported, we investigated possible allergenic differences between giant and short ragweed. To this end, we used 2 distinct approaches: a cross-sectional serological investigation of IgE reactivity to short ragweed in 3 distinct groups of patients allergic to ragweed, and a prospective *in vivo* investigation followed by the serological analysis of 1 specific case.

Cross-sectional serological investigation

Patients. Patients allergic to ragweed (all diagnosed on the basis of typical seasonal symptoms and positive SPT with giant ragweed

Blot-inhibition: Ragweed specific IgE

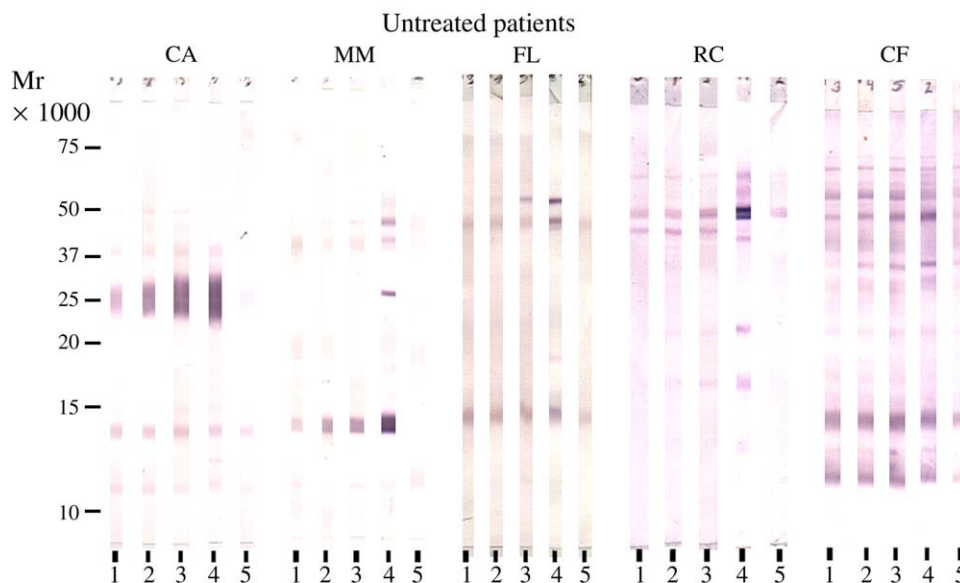


FIG 2. Inhibition of IgE antibody binding in a short ragweed immunoblot with sera from 5 subjects who had not undergone immunotherapy. Binding to short ragweed on the immunoblot was inhibited with (1) 0.9 mg giant ragweed extract, (2) 0.45 mg giant ragweed extract, (3) 0.23 mg giant ragweed extract, (4) no inhibitor, and (5) 1.0 mg short ragweed extract.

extract) cared for at this allergy unit in fall 2004 were asked to take part in this study. These subjects included patients already undergoing ragweed SIT as well as untreated patients. Sixteen patients agreed to undergo venopuncture and entered the study. These included 6 untreated patients, 4 giant ragweed injection SIT responders still undergoing giant ragweed SIT (Allergopharma, Reinbek, Germany), and 6 nonresponders to giant ragweed SIT who had been transferred to short ragweed SIT (Stallergènes, Anthony, France) with good results.

Allergen extraction, SDS-PAGE, and Western blotting. Investigations to detect IgE antibodies to short ragweed pollen were undertaken with sera from all subjects before and after absorption with either short or giant ragweed pollen extracts.

Pollen of short ragweed (*A. artemisiifolia*) or giant ragweed (*A. trifida*) delivered by Allergon (Engelholm, Sweden) was extracted as a 10 wt/vol suspension in 50 mmol/L PBS, pH 7.0, overnight at 2 to 8°C with slight agitation. Extractions were terminated by centrifugation (2500g × 20 minutes). Finally, supernatants (extracts) were passed through a 0.2-μm filter and stored at -20°C.

SDS-PAGE was performed by using NuPAGE 12% Bis-Tris gels (Invitrogen, Karlsruhe, Germany) with 2-(N-morpholino)ethanesulphonic acid/SDS electrophoresis buffer (Invitrogen). Samples (300 μg protein per gel, approximately 20 μg per strip) were separated under reducing conditions. After separation, proteins were transferred onto polyvinylidene difluoride membranes by semidry blotting.

In inhibition studies, sera of patients allergic to ragweed were preincubated with short or giant ragweed extracts in amounts as indicated (1.0-0.23 mg giant ragweed extract proteins, 0.9 mg short ragweed extract proteins, 100 μL serum; total volume, 1.0 mL) for 15 minutes at ambient temperature. Membrane strips bearing separated, and blotted short ragweed extract proteins were blocked by using 1% BSA and incubated with the mixtures of serum and inhibitor or sera without inhibitors overnight at ambient temperature. Bound specific IgE antibodies of sera with or without inhibitor were detected by incubation with an alkaline phosphatase conjugated

monoclonal antihuman-IgE antibody (Allergopharma) for 2 hours at ambient temperature and exposure to nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrate.

Prospective *in vivo* study

Patients. In spring, 2005 consecutive adult patients (age > 14 years) living in the area north of Milan, diagnosed as having ragweed allergy on the basis of both typical clinical symptoms (rhinoconjunctivitis with or without asthma from mid-August to the end of September) and positive SPT with short ragweed extract (Stallergènes), were considered eligible for this part of the study. To avoid the interference of minor, cross-reacting allergens such as profilin and calcium-binding proteins, only 41 subjects (M/F, 23/18) diagnosed as being monosensitized to ragweed were finally included; monosensitization was defined as the absence of any skin reactivity to seasonal airborne allergens other than ragweed, including grass, mugwort, pellitory, plantain, birch, olive, and cypress.

These 41 patients underwent comparative SPT with commercial extracts of both short ragweed (100 Index of reactivity [IR]/mL; Stallergènes) and giant ragweed (50,000 Biological Units [BU]/mL; Allergopharma). All SPTs were performed by using disposable 1-mm-tip sterile lancets (Stallergènes); readings were taken after 15 minutes, and reactions were expressed as mean wheal diameter (adding the longest diameter to the orthogonal diameter and dividing it by 2). A wheal diameter of 3 mm or more was considered positive.¹⁸ Histamine 10 mg/mL and saline were used as positive and negative control, respectively.

Western blot. Immunoblot analysis with short and giant ragweed pollen extract was performed with the serum from a 40-year-old woman allergic to ragweed (out of the former 41 ragweed monosensitized patients) that strongly reacted to short ragweed but did not show any reactivity to giant ragweed on SPT.

Pollen of short and giant ragweed was extracted as previously described. Electrophoresis of extracts (25 μg protein/lane) was performed in a 10% polyacrylamide precast gel (Nupage Bis-Tris,

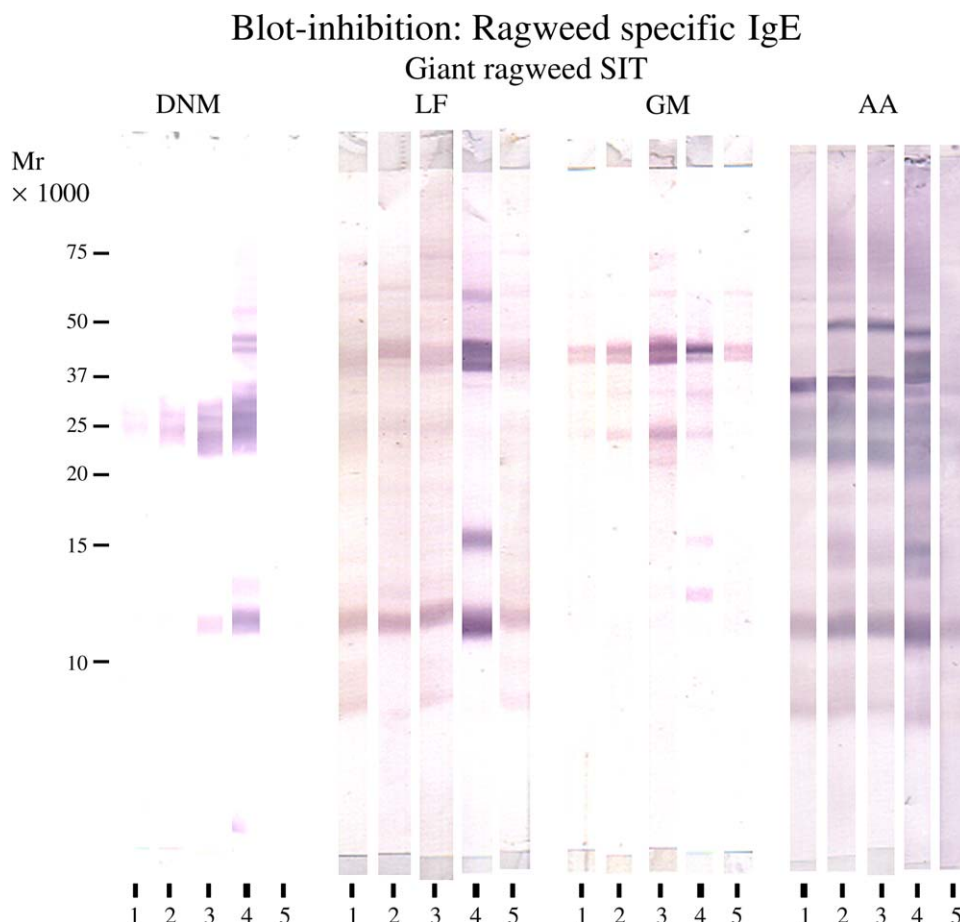


FIG 3. Inhibition of IgE antibody binding in a short ragweed immunoblot with sera from 4 subjects who had undergone immunotherapy with a giant ragweed preparation. Binding to short ragweed on the immunoblot was inhibited with (1) 0.9 mg giant ragweed extract, (2) 0.45 mg giant ragweed extract, (3) 0.23 mg giant ragweed extract, (4) no inhibitor, and (5) 1.0 mg short ragweed extract.

Novex; Prodotti Gianni, Milan, Italy) at 180 mA for 1 hour. The resolved proteins were transferred for 1 hour onto a nitrocellulose membrane according to Towbin et al.¹⁹ The membrane was saturated with 0.1 mol/L Tris-buffered saline containing 5% fat-free milk powder and incubated for 16 hours at 4°C with sera diluted 1:2. After 3 washings, bound specific IgE were detected by peroxidase-conjugated antihuman IgE antibodies from goat (1:1000 in saturation buffer) and by using an Enhanced Chemoluminescence Western blotting kit (Amersham, Milan, Italy) as substrate.

RESULTS

Serological analysis

Immunoblot analysis results are shown in Fig 1. Patients reacted to a large number of short ragweed allergens, although IgE reactivity at about 43 kd (corresponding to the molecular weight of major allergens Amb a 1 and Amb a 2) was present in nearly all cases.

Results of immunoblot inhibition studies are shown in Figs 2 to 4.

Among untreated patients (Fig 2), no difference in the extent of inhibition exerted by preincubating sera with

0.9 mg of giant ragweed or 1.0 short ragweed pollen was observed in patient FL. In contrast, patient CA showed a strong IgE reactivity at 25 kd that was only partially inhibited by giant ragweed extract, but totally inhibited by preabsorption with short ragweed extract. Similarly, patient MM IgE reactivity at about 12 kd, patient RC at about 40 kd, and patient CF reactivity at about 43 kd (probably the major allergen Amb a 1) were totally abolished by preabsorption with short ragweed, whereas preabsorption with giant ragweed extract caused only partial inhibition.

Among the 4 patients who responded to SIT with giant ragweed extract (Fig 3), no difference in inhibition exerted by giant and short ragweed was found in patients DNM, LF, and GM. Patient AA serum showed a marked IgE reactivity at about 35 kd that was not inhibited by giant ragweed but was totally inhibited by preabsorption of short ragweed.

Among the 6 patients who did not respond to giant ragweed SIT and were shifted to short ragweed SIT (Fig 4), IgE reactivity of sera from patients BS, BE, MP, and DCR against a 43-kd protein (most probably the major allergen

Blot-inhibition: Ragweed specific IgE

Giant ragweed followed by Short ragweed SIT

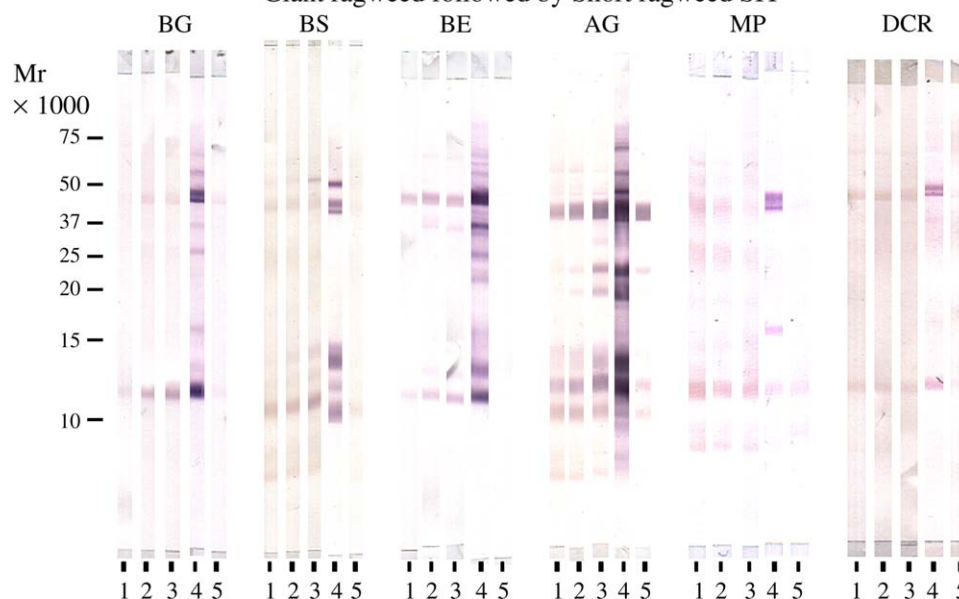


FIG 4. Inhibition of IgE antibody binding in a short ragweed immunoblot with sera from 6 subjects who were treated first with a giant ragweed preparation and subsequently with short ragweed immunotherapy. Binding to short ragweed on the immunoblot was inhibited with (1) 0.9 mg giant ragweed extract, (2) 0.45 mg giant ragweed extract, (3) 0.23 mg giant ragweed extract, (4) no inhibitor, and (5) 1.0 mg short ragweed extract.

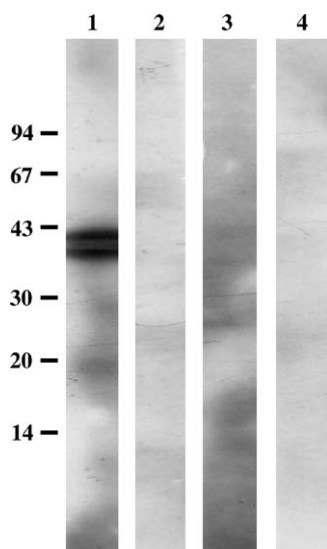


FIG 5. Ragweed specific IgE reactivity of serum from a short ragweed skin test-positive/giant ragweed skin test-negative subject. IgE reactivity of patient's serum (lane 1) and of a normal serum (lane 2) to *Ambrosia elatior* (short ragweed) pollen; IgE reactivity of patient's serum (lane 3) and a normal serum (lane 4) to *Ambrosia trifida* (giant ragweed) pollen.

Amb a 1) was totally inhibited by preabsorption with short ragweed but only partially inhibited by preabsorption with giant ragweed. Giant and short ragweed exerted an identical effect on serum from patients BG and AG.

Skin tests

Thirty-five of 41 patients were positive on SPT with both short and giant ragweed extracts; short ragweed induced a larger wheal in 19 of 35 (54%) cases, giant ragweed induced a larger wheal in 2 patients (6%), and the 2 extracts induced wheals of equivalent size in 14 patients.

Interestingly, 6 of 41 (15%) patients were strongly positive on SPT with short ragweed but negative on SPT with giant ragweed. The immunoblot analysis performed by using the serum from the only patient who accepted venopuncture confirmed *in vivo* observations (Fig 5). The serum showed a strong IgE reactivity at about 43 kD (most probably the major short ragweed allergen Amb a 1) but did not show any reactivity to giant ragweed.

DISCUSSION

Hitherto, short and giant ragweed have been considered as overlapping from an allergen point of view, but although earlier studies have shown strong cross-reactivity between group 1 and 2 group allergens,^{10,11} the minor group 5 allergen is a notable exception in that it shows little cross-reactivity between short and giant ragweed.¹³⁻¹⁷ Although not all reports totally agreed with this view, some older studies claimed that short and giant ragweed extracts were interchangeable during immunotherapy (see review²⁰). A recent review article from the United States confirmed that there is significant cross-reactivity between ragweed species, concluding that "selection of one species should

be sufficient for skin testing and immunotherapy.”²¹ The findings of the current study clearly bring this into question.

We started from the clinical observation that, in an area invaded by short ragweed but virtually giant ragweed-free, about 50% of patients did not respond to high-dose giant ragweed SIT, but responded very well if they were transferred to short ragweed SIT. Moreover, in the prospective part of the study, we demonstrated that SPT with short ragweed extract induced larger wheals than giant ragweed in the majority of patients, and that 15% of patients allergic to ragweed did not show any appreciable skin reactivity to giant ragweed in the face of strong sensitivity to short ragweed. All of these data pointed to an allergenic difference between short and giant ragweed. The rather high prevalence of patients allergic to ragweed who did not respond to giant ragweed immunotherapy seemed to rule out group 5 minor allergens, Amb a 5/Amb t 5. In effect, *in vitro* studies confirmed this hypothesis, showing that little or no IgE reactivity at 5 kd, the reported molecular weight of ragweed group 5 allergens,¹² was present in the study patients (Fig 1). Although allergenic differences between short and giant ragweed were observed with several allergens at different molecular weights, most of them were probably associated with the major allergens Amb a 1-2/Amb t 1-2 (43 kd) that are recognized by more than 90% of patients.¹² Importantly, the difference in IgE reactivity at 43 kd was observed particularly in patients who had been shifted to short ragweed SIT because of a poor response to giant ragweed SIT and, unequivocally, in the patient showing strong skin reactivity to short ragweed but negative on SPT with giant ragweed. The fact that all patients who did not respond to giant ragweed SIT were positive on SPT with giant ragweed along with the finding that preabsorption of sera with giant ragweed extract caused significant inhibition of short ragweed IgE reactivity in virtually all cases suggest that Amb a 1-2 and Amb t 1-2 differ only in a limited number of allergenic determinants, whereas most allergenic determinants are cross-reacting.

The results of this study have relevant practical implications because they suggest that in patients allergic to ragweed, both diagnostic procedures and immunotherapy should always be performed by using the ragweed species present in that specific geographic area.

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