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PHD THESIS

**THE ROLE OF RAGWEED POLLEN CALCIUM-BINDING PROTEINS
IN COMPARISON TO OTHER ALLERGENS IN THE MANAGEMENT
OF RAGWEED ALLERGY**

A B S T R A C T

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ABSTRACT

GENERAL PART

Allergy is a type 1 hypersensitivity reaction mediated by Immunoglobulin E (IgE) antibodies and the most common immune disorder affecting almost 30% of the population worldwide. World Allergy Organization (WAO) estimates that approximately 400 million individuals worldwide suffer from allergic rhinitis and 300 million suffer from asthma. Airborne allergens such as pollen and fungi are considered the most important air allergens.

Ragweed pollen is a major allergen source now distributed worldwide. Common ragweed (*Ambrosia artemisiifolia*) is a herbaceous weed, native to North America. Due to contaminated shipments of ragweed seeds from North America, ragweed underwent a major expansion in Europe at the beginning of the 20th century, with the Pannonian Plain being heavily invaded. Countries such as Hungary and Romania are most affected by ragweed dispersion. Ragweed pollen is highly allergenic and has the potency to induce allergic rhinitis and more severe symptoms such as asthma. Climate change, urbanization, and pollution have further contributed to the wide increased allergenicity of the ragweed pollen.

Currently, eleven allergens have been identified in the ragweed pollen. Two of these allergens are considered major, Amb a 1 and Amb a 11. More than 90% of the allergic patients are allergic to Amb a 1 whereas two-thirds may be reactive to Amb a 11. The rest of the allergens are minor with less than 50% IgE sensitization rate. Some of these allergens are important due to their ability to cross-react to other allergens within the same family of proteins. Amb a 6, Amb a 8, Amb a 9 and Amb a 10 are known pan-allergens from the ragweed pollen. Amb a 9 and Amb a 10 belong to the calcium-binding protein family and it is thought they may cross-react to Art v 5 from mugwort pollen, Phl p 7 from timothy grass pollen and Bet v 4 from birch pollen. So far, the ragweed allergy diagnosis is based on allergen extract and Amb a 1. Amb a 4 can be tested in multiplex systems with Amb a 1. However, the cross-reactive markers such as pan-allergens Amb a 9 and Amb a 10 are not included in the diagnosis.

Treatment of ragweed allergy includes avoidance of pollen exposure, symptomatic treatment and allergen immunotherapy (AIT). Avoidance of exposure is not feasible during pollen season and medication only treats the symptoms but not the disease. AIT is the only curative treatment able to modify the course of the disease with long-term effects. SCIT

(subcutaneous immunotherapy), also known as therapeutic vaccination, is the oldest and the most common form of AIT. However, the current AITs are based on ragweed pollen extracts. The drawbacks of the current extract-based AITs are the heterogeneity of the extract and the fact that allergen content is not standardized. Therefore using extracts could pose a problem due to pan-allergens which could lead to false positive results due to cross-reactivity. Moreover, the extracts could lack some allergens or they could be underrepresented. In addition to the issue of non-standardized allergen content, allergen extracts used for AIT are obtained differently by the manufacturers and thus the method of extraction, processing and denaturation may further affect their immunogenicity. Some extracts are modified to reduce allergenicity and maintain allergen immunogenicity, known as allergoids. This production-induced variability in the allergen content poses a problem for immunotherapy success. The problems regarding ragweed allergy diagnosis and treatment highlight the need for more studies on these topics.

SPECIAL PART

The present study aimed to improve the management of ragweed allergy, including the current molecular diagnosis and the immunotherapy treatment. The main aims of the current study were as follows:

- The first major objective was to determine the role of Amb a 9 and Amb a 10 allergens by identifying the IgE sensitization rate in a population from western Romania (Timiș county) and the possible cross-reactivity of these allergens with other similar allergens.
- The second major objective was to identify the sensitization patterns to major allergens and pan-allergens from ragweed, mugwort, birch and timothy grass pollen in a population from the same area by distinguishing the genuine sensitizing source from cross-reactivity.
- The third major objective was to evaluate registered AIT vaccines regarding the allergen-specific IgG induction and the efficacy of these IgG antibodies to block patients' IgE binding to the allergens.

To achieve the purposes of the present thesis, a clinically well-defined ragweed-allergic population consisting of 237 patients from western Romania was selected for these studies. Amb a 9 and Amb a 10 were recombinantly produced in *E. coli* and characterized to be further used to evaluate the IgE reactivity, clinical relevance and cross-reactivity. The IgE sensitization patterns to other allergen sources were determined using the available molecular diagnosis tools. Commercially available AIT vaccines were used to evaluate the immunogenicity of

ragweed pollen allergens by rabbit immunization and the blocking capacity of the induced IgG antibodies was tested in vitro.

The selection of ragweed-allergic subjects from western Romania was achieved at two Allergy Clinics in Timișoara, Romania. 150 patients were clinically characterized for one study and 87 for the second study. The experimental part of this study was carried out at the OncoGen Research Center within the "Pius Brînzeu" County Emergency Clinical Hospital, Timișoara, Romania. The animal study involving immunization of the rabbits with the recombinant allergens was carried out at the University of Agricultural Sciences and Veterinary Medicine "King Michael I of Romania" from Timișoara, Romania, whereas the immunization of rabbits with the registered AITs was performed by Davids Biotechnologie GmbH, Germany.

EXPRESSION AND PHYSICOCHEMICAL CHARACTERIZATION OF RAGWEED POLLEN CALCIUM-BINDING PROTEINS AMB A 9 AND AMB A 10

To characterize a population regarding IgE sensitization towards ragweed pollen calcium-binding proteins (CBPs) Amb a 9 and Amb a 10 were produced as recombinant allergens in the bacteria system (*Escherichia coli*) by genetic engineering. The first step in producing recombinant allergens was to select the sequence of the allergen of interest (Amb a 9.0101 and Amb a 10.0101). The construct used - pET27b plasmid - was modified by the ATG Biosyntetics to encode the sequence of Amb a 9 or Amb a 10, respectively. Heat shock administration transformed the construct in the *E. coli* BL-21-Gold cell line. The expression of the proteins was induced by the addition of the IPTG in the cell culture. The purifications of the recombinant allergens were carried out by nickel affinity chromatography using the six histidine tag (His-Tag) previously attached to the C-terminal end. After the purification, the proteins were dialyzed and the protein concentration was measured.

The proteins were further physicochemically characterized. The purity of the proteins was observed on the SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) under reducing and non-reducing conditions. Circular dichroism (CD) spectra and matrix-assisted laser desorption ionization - time-of-flight mass spectrometry (MALDI-TOF MS) were also carried out to obtain information regarding the secondary structure of the proteins and the molecular weight. The homology of the ragweed CBPs with other structures was also evaluated using the CLUSTAL Omega online tool to predict possible cross-reactive allergens.

rAmb a 9 obtained in the present study migrated to approximately 10 kDa (kilodaltons) on the SDS-PAGE whereas rAmb a 10 migrated to approximately 18 kDa and the calculated molecular weights calculated for Amb a 9 and Amb a 10 were confirmed in mass spectrometry

analysis (10 and 18 kDa). The results showed that Amb a 10 formed dimers and trimers on the gel under non-reducing conditions, but not Amb a 9, suggesting that Amb a 10 has the potential to be more allergenic. The circular dichroism was performed in the absence of calcium and revealed that the proteins contain a good amount of alpha-helix in their structures and the two proteins showed similarities in their secondary structure. The homology study revealed a good sequence identity of Amb a 9 with other 2 EF-hand CBPs from known allergen sources such as Art v 5, Syr v 3, Bet v 4, Ole e 3, Bra n 2, Che a 3, Phl p 7 and Cyn d 7 (approximately between 60-80%). Amb a 10, on the other hand, shares a limited identity sequence (50-60%) with the 4-EF hand protein Ole e 8 and other 2-EF hand proteins such as Bet v 4, Cyn d 7, Che a 3, Phl p 7, Syr v 3, Aln g 4.

THE ROLE OF RAGWEED POLLEN CALCIUM-BINDING PROTEINS IN RAGWEED ALLERGY

This study's main objectives were to identify the IgE sensitization to Amb a 9 and Amb a 10 in a population with an allergy to ragweed pollen from western Romania, to determine if these proteins possess clinical relevance for patients, and the ability to cross-react. Also, to obtain allergen-specific sera, two New Zealand White (NZW) female rabbits were injected with three doses of the allergens (rAmb a 9 or rAmb a 10), each of 200 µg using Freund's complete adjuvant (once) and incomplete adjuvant (twice) and immune sera were collected after the second and the third immunization.

First, the IgE reactivity towards rAmb a 9 and rAmb a 10 in ragweed-allergic patients from Western Romania (n=87) was tested by ELISA (enzyme-linked immunosorbent assay). The results indicated that one-quarter of the patients (25%) reacted to rAmb a 9 and approximately one-third (35%) reacted to Amb a 10. None of the patients showed monosensitization to ragweed CBPs, but sensitization to Amb a 1 indicated genuine ragweed allergy. The IgE sensitization rate to ragweed CBPs reported in this population was higher than previously reported, but the reactivity was not high.

Next, the allergenic potential of rAmb a 9 and rAmb a 10 to induce degranulation of the basophils, with the subsequent release of the chemical mediators, was determined in the mediator release assay. The mediator release assay is based on the rat basophils leukemia (RBL) cells transfected with the human high-affinity IgE receptor (FcεRI). RBL cells loaded with serum from six patients sensitized to ragweed CBPs and stimulated with six different allergen concentrations showed that rAmb a 10 induced degranulation in almost all patients positive to rAmb a 10 with the highest degranulation (i.e. 35%) at the maximum concentration

(1000 ng/ml). Amb a 9 seem to induce a lower degranulation in rAmb a 9-positive patient due to low IgE reactivity. Amb a 10 is a potent allergen but the induced degranulation is not high whereas Amb a 9 showed low allergenicity.

Furthermore, the association between different ragweed allergy phenotypes and sensitization to ragweed CBPs has been investigated. It was found that patients not sensitized to ragweed CBPs reported significantly more skin manifestations compared to patients sensitized to ragweed CBPs. These findings suggest that skin symptoms are not associated with CBP sensitization and could be associated with other ragweed allergens. Also, more patients sensitized to ragweed CBPs suffer most often from nasal, ocular and asthma symptoms.

The presence of cross-reactive epitopes between ragweed and mugwort CBPs was further evaluated using rabbit rAmb a 9-specific serum and rAmb a 10-specific serum in ELISA. The ELISA plates were coated with the allergens and pollen extracts of ragweed and mugwort and incubated with rabbit Amb a 9-specific serum and Amb a 10-specific serum and the reaction was detected with an anti-rabbit IgG antibody marked with an enzyme in the presence of a substrate solution. The results showed a similar trend with high reactivity to rAmb a 9 and rArt v 5 using rabbit rAmb a 9-specific sera and lower but considerable reactivity to rAmb a 10. rAmb a 10-specific serum bound rAmb a 10 better and showed a slightly lower and similar reactivity to rAmb a 9 and rArt v5. These findings suggest strong cross-reactivity behavior between these CBPs, especially between ragweed rAmb a 9 and mugwort rArt v 5. Furthermore, testing both rabbit allergen-specific sera towards ragweed and mugwort pollen extracts revealed low IgG reactivity overall, probably due to the low amount of CBPs in the extracts.

Although more studies are needed to confirm the clinical importance of Amb a 9 and Amb a 10, this study has shown that recombinant ragweed pollen calcium-binding proteins rAmb a 9 and rAmb a 10 have proven to be useful in the detection of specific IgE sensitization in ragweed-allergic patients and may be considered for the detection of sensitization to ragweed CBPs in the molecular diagnosis of ragweed allergy, among Amb a 1 and other ragweed allergens.

MAJOR ALLERGENS VERSUS PAN-ALLERGENS IN RAGWEED ALLERGY DIAGNOSIS

This prevalence study was conducted in western Romania investigating the IgE reactivity to different pollen sources in ragweed-allergic patients, aiming to detect the primary

sensitization, in order to provide better management of the allergic disease and to facilitate the prescription of AIT. This study examined sensitization profiles to major allergens and pan-allergens (profilin and polcalcins) from ragweed, mugwort, birch and timothy grass pollen.

First, the IgE reactivity of patients (n=150) to ragweed extract and allergens was tested in the Phadia ImmunoCAP platform. Besides ragweed pollen extract, the following allergens were tested: major ragweed pollen allergen (nAmb a 1) and major allergens from mugwort (nArt v 1), birch (rBet v 1), timothy grass (rPhl p 1/5b), as well as to profilins from ragweed (Amb a 8), mugwort (Art v 4), birch (rBet v 2), and timothy grass pollen (rPhl p 12) and to cross-reactive carbohydrate determinants (CCDs). The quantitative results are expressed in kUA/L and values ≥ 0.35 were considered positive. IgE reactivity to CBPs ragweed (Amb a 9 and Amb a 10), mugwort (Art v 5), birch (Bet v 4) and timothy grass pollen (Phl p 7) was tested in ELISA and partially in ImmunoCAP. The commercially available CAPs were obtained from Thermo Fischer Scientific and tested according to the manufacturer's protocol, whereas the allergens Amb a 8, Amb a 9, Amb a 10, Art v 4 and Art v 5 were manually coupled to the Streptavidin CAPs and patients' sera specific IgE was tested according to the manufacturer's protocol.

The results showed that 97% of the positive patients in the skin prick test (SPT) to ragweed extract were confirmed as genuinely sensitized to ragweed by ImmunoCAP to both ragweed pollen extract and Amb a 1. The IgE reactivity to Amb a 1 of more than 90% was consistent with that reported in the literature. The results showed that 19% of the patients had higher IgE reactivity to Amb a 1 compared to ragweed pollen extract. Moreover, for the majority of patients, it seems Amb a 1 plays a very important role in sensitization to ragweed pollen. On the other hand, in patients where Amb a 1 is responsible for less than 50% of the response to the whole extract, it may seem that other allergens also contribute to the IgE sensitization profile.

Next, the investigation of the sensitization profiles to other marker allergens from relevant allergen sources, such as Phl p 1/5, Art v 1 and Bet v 1 showed the most frequently recognized major allergen from other tested pollen sources was rPhl p 1/5b with an IgE reactivity of 29%, followed by Art v 1 with 18.7% and Bet v 1 with 4%. An important percentage of the ragweed-allergic patients (41.3%) included in this study showed multiple sensitizations. In addition to ragweed sensitization, 30% of patients reacted to one major allergen and 10% reacted to two other major allergens.

The sensitization to the before-mentioned sources appears not to be particularly high when considering the major allergens, but the profilins indicate a different story. The ragweed-

allergic patients were first tested to rAmb a 8 and revealed an IgE reactivity of 21%. Amb a 8-sensitized patients were further tested to the other profilins and all showed IgE sensitization to Art v 4 and Phl p 12, whereas 91% showed IgE reactivity to Bet v 4. None of the profilin-sensitized patients appear sensitized to all major allergens tested, indicating extensive cross-reactivity within the tested proteins. Previous studies indicated that the allergen source providing the highest profilin exposure could be responsible for the positive response to other profilins. In 56% of the profilin reactors, Amb a 8 is the culprit allergen in the Amb a 1-sensitized patients, in the lack of sensitization to major allergens from the other sources.

The sensitization to CBPs from ragweed pollen was also evaluated in this cohort study in ELISA and ImmunoCAP. The IgE reactivity to recombinant CPBs Amb a 9, Amb a 10 and Art v 5 was determined before by ELISA, therefore a quantitative measurement of the IgE levels to ragweed and mugwort CPBs was performed in ImmunoCAP for patients which showed reactivity to Amb a 9 and Amb a 10 in ELISA. Patients tested in ImmunoCAP showed low or no IgE binding to ragweed polcalcins nor to commercially available Bet v 4 and Phl p 7 nor to Art v 5. These results may be due to patients' low IgE levels to CPBs which were not detected in ImmunoCAP or perhaps ELISA is a more sensitive method able to detect low quantities of specific IgE. As stated before, the sensitization to CBPs may differ among ragweed-allergic patients. Many studies indicated Phl p 7 as well as Bet v 4 as good markers for the detection of polcalcin sensitization, but in this case, the cross-reactivity with ragweed CPBs could not be established due to the weak reactivity to ragweed CPBs.

When investigating the relationship between allergy symptoms and IgE sensitization, it was found that patients had a higher IgE reactivity to pollen extract compared to Amb a 1 regardless of the number or combination of symptoms the patients reported during the ragweed pollen season, indicating that other ragweed allergens play a role in inducing allergy symptoms. However, reactivity to pan-allergens Amb a 8, Amb a 9 and Amb a 10 could not be associated with any particular symptom.

For all CCD-positive patients, the allergen source responsible for sensitization was identified. Overall, based on the sensitization patterns to major allergens and pan-allergens, a primary sensitizing source could be identified in 57% of patients in this study cohort: in all but one, ragweed was the primary sensitizing source. One patient appears to be genuinely sensitized to mugwort and not to ragweed pollen.

In conclusion, the vast majority of patients exhibiting seasonal allergy symptoms and a positive SPT to ragweed pollen extract were genuinely ragweed-sensitized. The prevalence of sensitization to other major pollen allergens within this cohort of ragweed-allergic patients was

not high, but patients may appear positive in SPT to grasses, mugwort or birch pollen extracts due to profilin cross-reactivity. Thus, pan-allergens from the ragweed pollen should be included in component-resolved diagnostic (CRD) as cross-reactivity markers among the pan-allergens and marker allergens from other common allergen sources, to facilitate the correct identification of the culprit allergen and distinguish co-sensitization from cross-reactivity. These findings are a valuable contribution to the improvement of the CRD and also to the prescription of AIT.

EVALUATION OF EXTRACT-BASED AIT VACCINES FOR RAGWEED ALLERGY TREATMENT

This study aimed to evaluate the currently registered ragweed subcutaneous AIT vaccines available on the market by detecting the allergen-specific IgG titer against different ragweed pollen allergens and the ability of the induced IgG antibodies to block the patients' IgE binding to the allergen *in vitro*.

In this regard, two NZW female rabbits were immunized with one of the four AITs from different producers evaluated in this study. CLUSTOID (ROXALL Medizin, Vienna, Austria) is an allergoid containing aluminum hydroxide as an adjuvant. TYRO-SIT and POLLINEX (Bencard Allergie GmbH, Munich, Germany) are allergoids based on L-Tyrosin, with the addition of monophosphoryl lipid A (MPL) in POLLINEX, respectively. Diater (DIATER, Madrid, Spain) is an unmodified natural pollen extract based on aluminum hydroxide. The rabbits were immunized by Davids Biotechnologie GmbH (Regensburg, Germany) according to the manufacturer's immunization protocol for use in allergic patients. Blood samples were collected from the immunized rabbits before the first immunization (preimmune sera, PIS) every 4 weeks during the immunization (immune sera, IS) and the final blood samples were collected 4 weeks after the last administered injection (IS final).

The rabbit IgG titer against all ragweed allergens, such as Amb a 1 (rAmb a 1.01, nAmb a 1.01, rAmb a 1.03), rAmb a 3, rAmb a 4, rAmb a 5, rAmb a 6, rAmb a 8, rAmb a 9, rAmb a 10, rAmb a 11 and rAmb a 12 was detected by ELISA using sera from immunized rabbits diluted in six dilutions (1:100, 1:500, 1:1000, 1:5000, 1:10,000 and 1:50,000). The inhibition of the patients' IgE binding was performed in competition ELISA to Amb a 1, Amb a 4, Amb a 6, Amb a 8 and Amb a 11, considered major and/or relevant allergens. The allergens coated on the ELISA plates were incubated with rabbit IS, followed by the addition of serum from ragweed-allergic patients sensitized to these allergens. The inhibition of the allergenic activity was also performed in the RBL assay. The dialyzed rabbit sera were incubated with three

different allergen concentrations (1, 10 and 100 ng/mL) and added to the RBL cells previously loaded with patient sera.

The present study showed that these four AITs tested have induced different IgG profiles with respect to intensity and allergen specificity. Diater was able to induce the highest IgG response towards ragweed pollen extract in rabbits, but it also has the longest immunization protocol consisting of 14 injections of increasing doses over 4 months. Interestingly, the reactivity of the rabbit IS raised against ragweed pollen extract was most likely directed to Amb a 1 which showed similar IgG kinetics with the extract suggesting that Amb a 1 is the main elicitor of high sIgG titer upon immunization with Diater. Regarding other relevant allergens such as rAmb a 4, rAmb a 8, rAmb a 6 and rAmb a 11, as well as the minor allergen rAmb a 5, the administration of Diater showed different kinds of IgG profiles and kinetics to each of the allergens.

CLUSTOID was also able to induce a good IgG response towards Amb a 1, as well as to rAmb a 6 and rAmb a 10. However, immunization with CLUSTOID was able to induce a fast Amb a 1-specific IgG titer but the titer decreased one month after the end of initiation probably due to the short initiation protocol. Both TYRO-SIT and POLLINEX were able to induce Amb a 8- and Amb a 5-specific IgG antibodies, but only weak Amb a 1-specific IgG response.

The inhibition experiments showed that the IgE-blocking capacity was associated with high allergen-specific IgG levels in rabbit antisera. Diater-induced IgG antibodies were the most efficient in blocking patients' IgE binding to rAmb a 1, rAmb a 4, rAmb a 8 and rAmb a 11. CLUSTOID-specific IS at week 4 was also able to inhibit IgE binding to rAmb a 1.01 and to rAmb a 6, but to a lower degree in the final IS compared to sera collected at week 4. Moreover, a similar effect was observed using the before-mentioned sera in the mediator release assay. Diater-specific IS with high allergen-specific IgG titer inhibited the mediator release towards Amb a 1, Amb a 4 and Amb a 8 in all patients tested and CLUSTOID-specific sera inhibited IgE binding to Amb a 1 and to Amb a 6. Low IgG blocking ability was observed with POLLINEX, whereas no IgG blocking ability was observed with TYRO-SIT.

The response of rabbits to different allergen immunotherapies allowed comparison due to the final sera which was collected at the same endpoint, one month after the last injection of the initiation protocol. However, a reduced number of rabbits were used for each immunization and variation between rabbits responses can be found. Furthermore, when using other batches from the same manufacturers the results may differ due to the shortcomings of the allergen extracts. However, the initiation protocols for the AIT vaccination were highly variable in regard to the number of injections, the timeline of the immunizations and the applied

dosage. Moreover, the amount and concentration of the allergens in the extract itself as well as the immunogenicity of individual allergens also contributed to the variation in the induced IgG antibody titers. These findings underline the drawbacks of using allergen immunotherapy based on non-standardized commercial extracts. Furthermore, it highlights the need to develop new-generation immunotherapies for ragweed allergy treatment that reduce the side effects while containing the most relevant allergens able to induce blocking allergen-specific IgG antibodies that can compete with patients' IgE antibodies and reduce the occurrence of clinical symptoms as a result.

CONCLUSIONS

Based on the obtained results, the following can be concluded:

- Amb a 1 is indeed the most important ragweed pollen allergen inducing IgE sensitization in 97% or more of the Romanian population.
- Around one-quarter of the patients sensitized to ragweed pollen are reactive to calcium-binding proteins Amb a 9 and Amb a 10.
- Patients sensitized to ragweed pan-allergens (Amb a 8, Amb a 9, Amb a 10) may show sensitization to other sources due to pan-allergen cross-reactivity.
- Amb a 9 and Amb a 10 showed a strong recognition pattern to Art v 5 indicating cross-reactivity between these CBPs.
- Ragweed-allergic patients sensitized to Amb a 9 and Amb a 10 tend to report more often rhinitis, conjunctivitis and asthma-like symptoms, but not skin symptoms.
- Major allergens and pan-allergens are useful tools in distinguishing genuine sensitization from cross-reactivity, therefore Amb a 9 and Amb a 10 should be included in the CRD.
- Immunization of the rabbits with four different ragweed extract-based AIT vaccines induced different allergen-specific IgG levels towards ragweed pollen allergens.
- The 4 evaluated AIT vaccines differ in their allergen content, immunogenicity, dosage and length of the initiation protocol, which may affect the IgG induction.
- Diater vaccine based on natural unmodified pollen extract induced the best IgG response in rabbits compared to allergoids CLUSTOID, TYRO-SIT and POLLINEX.
- Elevated allergen-specific IgG levels induced upon rabbit immunization with the AIT vaccines were associated with good inhibition of patients' IgE binding.

- These findings represent the basis of personalized AIT selection containing the culprit allergen able to induce blocking IgG antibodies in allergic patients.
- The variation in the allergen content of the AIT vaccines as well as the cumbersome AIT immunization schedule highlight the need to develop next-generation AIT vaccines.

PERSONAL CONTRIBUTIONS

The following contributions were accomplished:

- Successful expression and production of the recombinant allergens Amb a 9 and Amb a 10 in the *Escherichia coli* system.
- Thoroughly characterization of Amb a 9 and Amb a 10, physicochemically as well as immunologically.
- Meticulous characterizations of the clinical symptoms of 237 ragweed-allergic patients from western Romania.
- Successful coupling and testing for specific IgE of the recombinant Amb a 8, Amb a 9 and Amb a 10, Art v 4 and Art v 5 for the first time by ImmunoCAP.
- Identification of the genuine sensitizing source in 150 patients reactive to allergens from ragweed, mugwort, birch and timothy grass pollen.
- Evaluation and comparison of different ragweed extract-based AIT vaccines in animal models for the first time in the literature.
- Inhibition of the basophils mediator release towards ragweed allergens achieved in the RBL assay with antisera from rabbits immunized with ragweed AIT vaccines.