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PHD THESIS

**IMMUNOPROTEOMIC CHARACTERIZATION OF RECOMBINANT
RAGWEED POLLEN ENOLASE AND NSLTP AND THEIR CLINICAL
RELEVANCE**

A B S T R A C T

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ABSTRACT

Allergic diseases are health conditions of increasing worldwide prevalence, especially in countries with westernized lifestyle. They are characterized by an exaggerated immune response towards harmless environmental stimuli, most frequently proteins. In western and southern Romania, ragweed pollen has become a serious health concern due to the rapid spread of the plant, the high pollen loads and increased allergenicity of the pollen. This study **aimed** to evaluate the role of two different minor allergens in ragweed pollen allergy, the recently discovered enolase, Amb a 12 and the non-specific lipid transfer protein, Amb a 6. The characterization required the production of pure, quantifiable allergen molecules. These proteins were then evaluated regarding their physicochemical properties, i.e. mass spectrometry measurement, quantification of secondary structure content, formation of aggregates. Additionally, the IgE binding frequency was evaluated as well as the allergenicity among 150 ragweed pollen allergic patients. Allergen-specific antibodies were also generated by rabbit immunizations to quantify the allergen in ragweed pollen extract. The antibodies were also used to screen various pollen and food allergen sources for similar proteins, which could possibly induce cross-reactive IgE responses. Upon identification of the relevant allergens, peptides covering the allergen sequence were designed to identify common epitopes between allergens from the same allergen family. These peptides also aided in mapping the IgE epitopes and could serve as a basis for further development of specific allergen immunotherapies (AIT).

General part

The immune defense is categorized into three cellular responses which work against different pathogens, type I responses are directed against intracellular pathogens, type II response against parasites and type III responses against extracellular pathogens. T lymphocytes and their differentiation are the key players regulating these responses. Malfunctions within these cellular responses result in allergic and autoimmune diseases. Distinction between different phenotypes of these disorders have led to the definition of nine types of hypersensitivity responses. Based on the main antigen responders types I-III hypersensitivities are defined as antibody responses, types IV_{a-c} are induced by cellular

responses, type V and VI are tissue responses towards antigens, whereas type VII hypersensitivity results from direct contact with the antigen. The mechanism involved in different types of hypersensitivity give rise to the manifestations of allergic diseases. However, the heightened immune response towards harmless environmental proteins often results from the interplay between type II hypersensitivity response mediated by immunoglobulin E (IgE) antibodies and type IV_b hypersensitivity by the differentiation of T lymphocytes towards type 2 helper cells (Th2). The allergic response is divided into two different phases, the sensitization phase, during which the antigen is first taken up by antigen presenting cells (APCs) and presented to T cells. Under stimulation with type 2 cytokines, i.e. IL-4, the T lymphocytes differentiate into type 2 helper cells. These in turn present the antigen to B cells and, under IL-4 and IL-13 stimulation, trigger a class switching in B cells. This class switch induces the differentiation of B cells into IgE producing plasma B cells. The IgE antibodies can bind to the high affinity IgE receptor (FcεRI) on the surface of effector cells – basophils and mast cells – resulting in their sensitization towards the antigen. The second phase of an allergic response is the effector phase. During this phase the antigen is now recognized by the effector cells as an allergen. Once the allergen binds to the IgE receptor on the surface of basophils and mast cells, it triggers the cross-linking of the FcεRI and the release of inflammatory mediators which result in the symptoms associated with allergic disease. The low affinity IgE receptor (CD23) also plays a role in the regulation of allergic diseases, especially in food allergies, by its presence on the surface of intestinal epithelial cells. The cross-linking of this receptor can transport complexes formed by the allergen and IgE antibodies across the epithelial barrier, where the complexes can bind to sensitized mast cells and trigger an inflammatory response.

Allergic diseases are often in response to environmental proteins, including house dust mites, pollen and insect venoms. The clinical manifestations of allergy include atopic dermatitis, eczema, allergic rhino-conjunctivitis and asthma, but also anaphylaxis in response to certain food or insect venom allergens. A family history of allergies often results in infants also developing these conditions, especially if the mother has the disease. This has led to the conclusion that allergic diseases result from an interplay between genetic and environmental factors. The key players in the development of allergic diseases include dysfunctions in the epithelial barrier, the differentiation of T cells shifted towards Th2 cells and the production of IgE antibodies. Genetic factors which predispose to allergic diseases include single nucleotide

polymorphisms in genes coding for proteins involved in the barrier function of the epithelium or for molecules related to antigen presentation, i.e. loss of function mutations of filaggrin, mutation in major histocompatibility complex. Loss of function of filaggrin mutation was found to predispose towards the development of atopic dermatitis. Additionally, certain alleles of antigen presenting molecules of the class II major histocompatibility complex were predisposed towards the presentation of certain inhalant allergen molecules, favoring allergen sensitization. Environmental factors were also associated with allergic diseases, either by directly influencing the key components, i.e. inducing the production of type 2 inflammatory cytokines or disrupting the epithelial barrier. Indirectly, environmental factors can induce epigenetic modifications which regulate the expression of genes involved in the epithelial barrier or the differentiation of T cells. These epigenetic modifications are comprised of DNA methylations, methylation, acetylation or ubiquitination of histones or microRNA (miRNA) which either increase or decrease the expression of genes involved in T cell differentiation by their occurrence in promotor regions. Additionally, DNA methylations were found to increase to activation of cells involved in the allergenic immune response, i.e. eosinophils and natural killer cells. These changes in gene expression were associated with asthma development in childhood. The maternal environment was also found to contribute to the development of allergic diseases in infants. Uncontrolled asthma and smoking were identified as factors predisposing to asthma development. Maternal allergic diseases would also create the immunological microenvironment, priming the immune response of the fetus. An important environmental factor in maternal allergy would be maternal IgE. These antibodies were found to be transferred to the fetus via the umbilical cord, crossing the placental barrier. On fetal mast cells, the fetal low affinity IgE receptor is able to bind IgE and/or IgE-allergen complexes, thereby becoming sensitized to an allergen, similar to the sensitization in food allergies. Protective factors in the development of allergic diseases were found to be sub-pathogenic microbial infections which could shift the differentiation of T lymphocytes towards type 1 helper T cells (Th1). In a similar fashion, prenatal immunization with allergen immunotherapy was considered to have a protective role against allergy development. Additionally, maternal nutrition was found to have a protective effect, including diets consisting of fresh fruit, vegetables and yogurt, as well as supplementation with vitamin D and intake of omega-3 fatty acids during pregnancy.

Allergic diseases show a progression from skin symptoms, like atopic dermatitis or eczema in infancy and childhood towards respiratory symptoms, like allergic rhinitis or asthma in adolescence. These are accompanied by IgE sensitization towards food allergens in childhood and respiratory allergens in adolescence and adulthood. This progression is known as atopic march. However, seven different trajectories have been identified based on the onset, severity, persistence and co-occurrence of skin and respiratory symptoms and IgE sensitizations. The sensitizations are considered to occur in the order of encounter with the allergens. Therefore, in infancy and childhood sensitizations occur in response to hen's egg and cow's milk. The food sensitizations start to decrease in childhood, being replaced by respiratory sensitizations. Among the first respiratory allergens, house dust mite sensitization starts to occur in childhood. At school age, pollen sensitizations start to develop, which lead to symptoms of allergic rhinitis and asthma in adolescence. Multiple sensitizations are often associated with more severe or more persistent symptoms, with early sensitization to certain risk molecules, i.e. Fel d 1 from cat dander or Phl p 4 from timothy grass pollen, acting as indicators of respiratory allergy in adolescence.

Ragweed pollen allergy has become an increasing health problem throughout the distribution area of this plant. The decreased use of agricultural surfaces in addition to climate change and urbanization have contributed to greater dispersal and higher availability of suitable habitats for this invasive plant species. Additionally, increasing temperatures and pollution contribute to a higher production of pollen of increased allergenicity. Different climate change scenarios also predict the expansion of the species further north and towards higher altitudes, resulting in prolonged exposure towards this pollen, increasing IgE sensitizations and more persistent and severe symptoms among already sensitized patients. Additionally, particulate matter resulting from pollution was found to act as a depot and carrier of allergens. Increased levels of NO₂ were found to induce upregulation of certain allergens. In ragweed pollen, eleven allergens have been identified so far. Amb a 1 and Amb a 11 are considered major allergens according to their rate of IgE binding. Among the minor allergens, polcalcins Amb a 9 and Amb a 10, profilin Amb a 8, and non-specific lipid transfer protein Amb a 6 are considered pan-allergens, due to their prevalence in multiple plant tissues and species. Hence these proteins show varying degrees of cross-reactivity, especially with the homologues from mugwort pollen. Despite previous considerations, that Amb a 1 would suffice for the accurate

diagnosis and treatment of ragweed pollen allergy, recent findings highlight that up to 10% of ragweed allergic patients do not show specific IgE (sIgE) towards this allergen. Patients show complex IgE sensitization patterns, wherein a higher number of symptoms or more severe symptoms (asthma) are associated with sensitization to other allergens in addition to Amb a 1. The investigation of the IgE sensitization profiles in common ragweed by means of component resolved diagnosis would provide further insights into the role of individual allergens in ragweed pollen allergy. Component resolved diagnosis would offer the possibility to identify sensitization to possible risk molecules predicting the progression of the allergic disease. Testing the IgE reactivity towards individual components could facilitate the identification of genuine sensitization compared to cross-reactivity induced by pan-allergens. Another benefit includes the possibility of a personalized treatment of allergy based on the sensitization profiles, indications regarding the immunotherapy prescription and the monitorization of the production of protective antibodies upon administration of allergen immunotherapy.

Exposure of common ragweed to elevated levels of NO₂ induced the upregulation of a novel allergen with high sequence identity with rubber tree enolase, Hev b 9. Enolase is an enzyme involved in glycolysis, indicating the importance of the sequence conservation to maintain its function. Thus, the sequence identity between different allergenic enolases was relatively high, 60% between plant and animal enolases with fungal enolases. The enolase domain was conserved between Amb a 12 and the homologues from rubber tree, yellowfin tuna and cockroach. These identities were reflected in the phylogeny, plant and animal enolases forming a separate clade from fungal enolases. In mold allergy, enolases were identified as frequently cross-reactive allergens, with *Alternaria alternata* Alt a 6 and *Aspergillus fumigatus* Asp f 22 as important allergens. Allergenic enolases were also found in yellowfin tuna Thu a 2, cockroach putative Per a and rubber tree, Hev b 9. The enolase IgE sensitization rate was low in these allergen sources ranging between 14% and 30%. Higher sensitization rates were detected in certain patient groups, i.e. 30% sensitization to Pen c 2 among asthmatic patients. Additionally, antibodies against enolases have been involved in the development of autoimmune diseases.

Non-specific lipid transfer protein, Amb a 6 is a pathogenesis-related protein, a protein family which has been involved in the induction of severe symptoms in the mediterranean region, mostly driven by sensitization to peach Pru p 3. The protein structure is stabilized by

disulfide bridges, conferring resistance towards heat treatment and enzymatic digestion. This structural stability was considered responsible for the induction of severe symptoms in patients sensitized towards proteins from this protein family. nsLTPs are found in most plant tissues and are considered responsible for the induction of cross-reactive IgE responses, especially among different Rosaceae species. These proteins are major allergens in both food and respiratory allergens, Pru p 3 in peach and Par j 1, Par j 2 in wall pellitory pollen. In these sources, they have been associated with induction of severe symptoms, from anaphylaxis to asthma. Also, Tri a 14 from wheat flour has been associated with the development of an occupational allergy, baker's asthma. The severity of the reaction towards nsLTP was also found to be influenced by co-factors, like non-steroidal anti-inflammatory drugs or exercise. The sequence identity between different nsLTPs is below the level expected to induce cross-reactive IgE responses, ranging between 30% and 50%, being higher among closely related plant species, i.e. peach, apple, cherry and hazelnut. The sensitization rate towards nsLTPs was around 28% among patients with food or respiratory allergy in an Italian cohort. Peach nsLTP was the most frequent sensitizer in food allergy and Par j 2 in allergic asthma patients. While Amb a 6, *Helianthus annuus* and wall pellitory nsLTP form a clade, Art v 3 from mugwort does not cluster with the other weed pollen nsLTPs. Very few regions in the protein sequence appear conserved among different allergenic nsLTPs, only the cysteine residues showing a high degree of conservation. Both Amb a 6 and Amb a 12 were described as allergens in common ragweed pollen, yet an in-depth characterization of these allergens, as well as the association of sensitization towards these allergens and clinical features are still missing.

The spread of common ragweed, the increasing allergenicity of the pollen and the importance of component resolved diagnosis for the accurate diagnosis and treatment of allergic patients highlight the importance of investigating the role ragweed pollen enolase and nsLTP play in ragweed pollen allergy.

Special part

Physicochemical characterization of recombinant allergens

A prerequisite for the investigation of the relevance of individual allergens within an allergen source, is the availability of pure, quantifiable allergens. This can be achieved by recombinant allergen production. Therefore, recombinant ragweed pollen enolase and nsLTP

were produced in different protein expression system. A gene construct was designed for both proteins including a hexa-histidine tag at the N-terminus to facilitate purification and identification. Amb a 12 was produced in *Escherichia coli* cells (eAmb a 12) as well as *Spodoptera frugiperda* Sf9 insect cells (iAmb a 12). Amb a 6 had been successfully produced in a eukaryotic expression system (yeast) and the high number of cysteine residues indicated better suitability of the eukaryotic expression system, i.e. Sf9 cells (rAmb a 6). Small scale expression experiments were performed to determine the optimal expression conditions, eAmb a 12 after overnight expression, iAmb a 12 and rAmb a 6 were obtained after 96h incubation with the baculoviral stock. The proteins were isolated and purified using affinity chromatography. The molecular weight of the proteins, their purity and the formation of oligomers were verified on SDS-PAGE. Both proteins formed dimers on SDS-PAGE when separated under non-reducing conditions. Amb a 12 migrated close to 55 kDa and Amb a 6 around 15 kDa under reducing conditions. Structures identified as dimers by immunoblotting with His-tag detection were found close to 130 kDa for Amb a 12 and close to 28 kDa for rAmb a 6. The ability of the proteins to bind IgE were further verified by immunoblotting with serum from reactive patients. eAmb a 12 and iAmb a 12 induced IgE binding on immunoblot mostly towards the monomeric form of the protein, separating under reducing condition. rAmb a 6 induced stronger IgE binding by separation under non-reducing conditions, indicating the presence of conformational epitopes. The molecular weight of the proteins determined by mass spectrometry was 48.8 kDa for eAmb a 12, 48.9 kDa for iAmb a 12 and 10.9 kDa for rAmb a 6. These molecular weights are in agreement with the expected mass based on the protein sequence. Gel filtration confirmed the dimer formation of iAmb a 12 and rAmb a 6. In terms of secondary structure content, the proteins consisted mostly of alpha-helical structures, i.e. 32% for iAmb a 12 and 54% for rAmb a 6. eAmb a 12 had a higher proportion of beta-sheets which was diverged from the structure of the reference enolase and the determined 3D model of the protein. Enzymatic activity was highest for the reference enolase, followed by iAmb a 12 and lowest for eAmb a 12. The formation of dimers was identified as an important factor in the enzymatic activity of the enolases, possibly contributing to the higher enzymatic activity of iAmb a 12.

IgE binding frequency and possible cross-reactive allergen sources

The IgE binding frequency was determined in ELISA for the ragweed enolase and in ImmunoCAP for ragweed nsLTP. The IgE binding frequency among 150 ragweed pollen allergic patients was 15% for eAmb a 12 and 19% for iAmb a 12, whereas rAmb a 6 bound IgE in 30% of ragweed allergic patients. Thereby, both allergens can be considered minor allergens based on their IgE recognition frequency. The levels of IgE were relatively low towards ragweed pollen enolase, whereas rAmb a 6 induced high levels of sIgE which were comparable to the levels induced by the major allergen Amb a 1 for a few patients. IgG antibodies recognizing the recombinant allergens were produced by rabbit immunizations. These were then used for screening common pollen (ragweed, mugwort, birch, timothy grass, olive and wall pellitory) or food (apple peel, pulp, banana, kiwi, peach peel, pulp, peanut and wheat flour) allergen extracts for possible cross-reactive proteins. Rabbit Amb a 12-specific IgG antibodies responded in immunoblot towards most of the pollen and food allergen extracts tested, except for wheat flour. In ELISA the response was also similar, IgG reactivity being detected even in wheat flour, yet overall the response was lower towards food than towards pollen allergen extracts. For further investigation of the clinical relevance of these potential cross-reactivities, pollen and food allergen extracts were tested regarding their ability to inhibit IgE binding towards eAmb a 12 and iAmb a 12 using serum from five enolase-reactive patients. Herein, especially peach pulp extract and kiwi extract managed to inhibit between 30% and 44% of IgE binding towards iAmb a 12 and eAmb a 12. IgE inhibition towards ragweed enolases using pollen allergen extracts was close to 0%. This could be attributed to a low concentration of enolases in the pollen extracts. There were individual difference in IgE inhibition with allergen extracts, in one patient peanut extract managing to inhibit up to 60% of IgE binding towards the ragweed enolases. Contrary to the extensive co-recognition of enolases among different extracts, rAmb a 6-IgG was detected only towards rAmb a 6, Par j 2 and ragweed pollen extract. A similar pattern of IgG reactivity was achieved with Par j 2-specific serum and the allergens. These rabbit antibodies managed to inhibit on average 72% of IgE binding towards rAmb a 6 among twelve nsLTP-reactive patients, IgE inhibition ranging between 35 and 95%, depending on the titer of IgE antibodies.

Allergenicity and association with clinical features

The allergenic activity of the recombinant proteins was also tested in terms of the mediator release (β -hexosaminidase) induced by the allergens in humanized rat basophil leukemia cells (huRBL) primed with serum from enolase or nsLTP-reactive patients. For control purposes the cells were also stimulated with the major allergen, nAmb a 1.01. The β -hexosaminidase released in response to Amb a 12 was overall low, exceeding 30% only in two of the ten patients tested. No differences were noted between eAmb a 12 and iAmb a 12. nAmb a 1.01 managed to induce up to 90% mediator release in four of the tested patients. Allergenic activity was also evaluated upon stimulation with rAmb a 6 and Par j 2 as a potentially cross-reactive nsLTP in huRBL cells primed with serum from eight nsLTP-reactive patients. rAmb a 6 managed to induce up to 100% β -hexosaminidase release in cells primed with serum from one of the reactive patients. Up to 40% mediator release in response to rAmb a 6 was detected in huRBL cells primed with serum from one patient which was negative towards nAmb a 1.01. In five of the tested patients, the β -hexosaminidase release in response to rAmb a 6 was higher than towards the major allergen nAmb a 1.01. Par j 2 did not induce mediator release in huRBL cells primed with serum from any of the rAmb a 6-reactive patients tested.

The association of reactivity towards ragweed pollen enolase and nsLTP with other clinical features was also investigated, i.e. diagnosis, reported symptoms, number of symptoms or other sensitizations. There were no significant differences among the reported symptoms related to Amb a 12 or Amb a 6 reactivity, nasal symptoms and ocular symptoms having been most frequently reported. Skin symptoms were more frequently reported among Amb a 12-reactive patients, with urticaria diagnosis occurring in a higher proportion of Amb a 12-reactive patients (10.34% compared to 4.13%). The higher occurrence of skin symptoms could be indicative of a different route of sensitization through the skin, as has been suggested for Der p 11, a marker for atopic dermatitis among house dust mite allergic patients. Rhinorrhea and wheezing were more frequently reported among rAmb a 6-reactive patients. This was reflected in a trend towards a higher proportion of nsLTP-reactive patients reporting asthma-like symptoms in addition to nasal and ocular symptoms (48.8% compared to 35.24%). The differences were not statistically significant.

The higher proportion of skin symptoms among Amb a 12-reactive patients could also have resulted from a higher rate of food allergy among enolase-reactive patients (four patients among Amb a 12-reactive group compared to only one patients in the Amb a 12-non-reactive group). However, no particular food allergen was common for all four food allergic patients, making a clear conclusion related to possible sources of cross-reactive allergens difficult. No particular inhalant allergen sensitization was detected more frequently in relation to Amb a 12 reactivity. Despite the known cross-reactivity among unrelated nsLTPs, only one of the food-allergic patients was also reactive to rAmb a 6. The low sequence identity towards other allergenic nsLTPs, the lack of mediator release in response to Par j 2 as well as the low probability nsLTP-related food allergy hint towards the pollen-specificity of this allergen. The high sIgE levels and mediator release among reactive patients also indicated the clinical relevance of rAmb a 6 in ragweed pollen allergy.

IgE epitope mapping using Amb a 6-derived peptides

The high clinical relevance of Amb a 6 prompted more in-depth investigations relating to the IgE binding sites of this protein. Therefore, four peptides were designed covering the sequence of Amb a 6, excluding highly hydrophobic regions. The peptides were conjugated with keyhole limpet hemocyanin (KLH) to induce peptide-specific antibodies by rabbit immunization. The IgE binding ability of these peptides was investigated in addition to their ability to induce mediator release in huRBL cells primed with serum from Amb a 6-reactive patients. The peptides did not show significant IgE binding among the seven nsLTP-reactive patients tested. Only the two peptides from the C-terminus, A6-3 and A6-4, showed slight IgE reactivity in two of the tested patients, which was a lot lower than towards rAmb a 6. Neither the unconjugated, nor the conjugated peptides induced β -hexosaminidase release in huRBL cells primed with serum from six rAmb a 6-reactive patients. Only the peptide from the N-terminus, A6-1, induced mediator release at the highest concentration, yet this could be an effect of the buffer used to solubilize the peptide, 35% formic acid lysing the basophils. The IgG reactivity of the peptide-specific serum towards rAmb a 6 was investigated in ELISA. The same method was used to test the IgG reactivity of the peptide-specific serum towards Art v 3, the nsLTP from mugwort, and Par j 1, the nsLTP from wall pellitory pollen. The Amb a 6-specific serum showed a high IgG reactivity towards Par j 1 and towards the peptides A6-2

and A6-4. A6-1-specific serum showed IgG reactivity towards Par j 1 and A6-4-specific serum towards Art v 3. The ability of these peptide-specific antibodies to inhibit IgE binding among sixteen Amb a 6-reactive patients was investigated in ELISA. The IgE inhibition of the individual peptide-specific serum and of the combined peptide-specific sera did not reach the inhibition achieved with the allergen-specific serum (maximum inhibition with allergen-specific serum 90.6% versus combined peptide-specific serum 44.4%). This would be indicative of potential conformational epitopes which have not been covered by the sequential peptides.

Conclusions

Ragweed pollen enolase and nsLTP were successfully produced as recombinant proteins. The purified proteins were folded and managed to bind IgE among patients with diagnosed ragweed pollen allergy. Both allergens can be considered minor allergens based on the IgE recognition frequency. However, differences were noticeable regarding their allergenicity. Amb a 12 lacked or showed only reduced ability to trigger mediator release in huRBL primed with serum from allergic patients. The sequence identity of Amb a 12 with other allergenic enolases was high. Thus, proteins of similar molecular weight to Amb a 12 were detected in most of the food and pollen allergen extracts tested, with peach pulp and kiwi extracts showing the highest ability to inhibit IgE binding towards Amb a 12. The association with clinical features revealed that a high proportion of food allergic patients had IgE reactivity towards Amb a 12, although no common food allergen source was identified among the reactive patients. This would be in agreement with the individual differences found in the inhibition of IgE binding using allergen extracts. Amb a 12-reactive patients tended to be more often diagnosed with urticaria, which could be due to the higher prevalence of food allergy in these patients. Insect cell expressed Amb a 6 was a protein with a high proportion of alpha-helix sheets. The protein separated under non-reducing conditions showed better IgE binding than under reducing conditions indicating the presence of conformational IgE epitopes. This was supported by the results with the peptide-specific sera. The IgE binding frequency confirmed that Amb a 6 is a minor allergen. However, the allergenicity assay and the high sIgE levels comparable to those for the major allergen Amb a 1 among some sensitized patients showed that Amb a 6 is relevant allergen in ragweed pollen allergy. The rabbit Amb a 6-specific antiserum revealed low reactivity with other pollen extracts, yet some IgG binding

towards the major allergens from wall pellitory. The reactivity towards the wall pellitory allergens was not reflected in the functional assay, cells primed with serum from Amb a 6-reactive patients did not degranulate in response to Par j 2. Contrary to previous findings which reported cross-reactivity between pollen and food nsLTPs, only one Amb a 6-reactive patient was allergic to plant-based foods. Also, the IgG reactivity towards Art v 3 from mugwort pollen was low. Amb a 6-derived peptides did not induce mediator release in huRBL cells, and had almost no IgE binding. Thus, Amb a 6 appears to be a pollen specific nsLTP, showing high IgE reactivity, capable of inducing mediator release. The Amb a 6-derived peptide specific serum did inhibit IgE binding towards the allergen, yet only half to the extent of rAmb a 6-specific serum, indicating the presence of conformational epitopes.

The role of Amb a 12 as a minor ragweed pollen allergen has been confirmed in this study, as well as its potential for cross-reactivity with multiple proteins found in allergenic foods. Although Amb a 6 was identified as an important allergen within this cohort of ragweed pollen allergic patients, testing the reactivity among different populations would provide further evidence regarding the relevance of this allergen in ragweed pollen allergy. The study could have also benefited from a more detailed structural characterization of the allergens, like measurement of the near UV spectrum to gain insights into the arrangement of aromatic side chains and the tertiary and quaternary structure of the protein. Additionally, X-ray crystallography studies would provide an accurate structural characterization of the protein. The design of additional peptides, potentially covering conformational epitopes could provide additional information on the IgE binding regions. The identification of further relevant ragweed pollen allergens and the IgE binding sites of these allergens could be used to design a next generation AITs based on allergen peptides. This would require additional testing of the safety and efficacy of allergen-derived peptides included in this study, i.e. T cell proliferation assays. Further selection of all clinically relevant peptides to be included in the design of peptide-based AITs would then also require testing the IgE reactivity, allergenicity and the T-cell proliferation ability of the peptide constructs.

PERSONAL CONTRIBUTIONS

- Literature review on the hypersensitivity mechanism and prenatal factors in the development of allergic diseases
- Retrieval of allergen sequences, protein Blast, identity matrix construction and construction of phylogenetic tree for the investigated ragweed allergens
- Cultivation of *Escherichia coli*, *Spodoptera frugiperda* (Sf9) and humanized rat basophil leukemia cells (huRBL)
- Construct design for recombinant allergen production
- Small scale expression experiments to determine optimal expression conditions
- Transformation, transfection, protein isolation and purification
- Preparation of pollen and food allergen extracts
- Quantification of purified allergens in the allergen extracts
- Immunoblotting of recombinant allergens
- Testing enzymatic activity of recombinant allergens Amb a 12
- Testing of IgE reactivity in ELISA, biotinylation of recombinant allergens and quantification of sIgE in ImmunoCAP
- Evaluation of allergenicity of recombinant allergens
- Evaluation of association between IgE reactivity and clinical phenotype
- Determination of relative prevalence of allergens within allergen extracts
- Evaluation of potential cross-reactive proteins within pollen and food allergen extracts
- Coupling of peptides covering the sequence of Amb a 6
- Evaluation of IgE reactivity and allergenicity of peptides
- Testing the cross-reactivity of peptide-specific serum with allergenic non-specific lipid transfer proteins from mugwort and wall pellitory
- Evaluation of IgE binding inhibition of individual and combined peptide-specific serum