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DOCTORAL THESIS

PHYSICOCHEMICAL AND IMMUNOLOGICAL CHARACTERIZATION OF AMBROSIA ARTEMISIIFOLIA MAJOR ALLERGEN AMB A 11, WITH CLINICAL CORRELATIONS

DISSERTATION ABSTRACT

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INTRODUCTION

The importance of the chosen research theme results from the spread of ragweed in Europe and Romania, associated with the increased load of atmospheric pollen during the pollen season and severe allergic disease manifestations in the exposed population.

Ragweed (Ambrosia artemisiifolia) is a plant native to the USA, where in 1930 it was estimated that more than 3 million people were sick with ragweed-induced hay fever. At the beginning of the 20th century, the first plant control programs were initiated, after a few decades its important role in the development of respiratory allergies (hay fever and asthma) was also recognized. More recently, the 2005-2006 NHANES study concluded that up to 15% of Americans tested positive for ragweed pollen-specific IgE.

In Europe, the oldest mentions of ragweed were found in the herbaria of botanical gardens (Lyon 1763, Paris 1775), but the hypothesis of the spread of the plant from botanical gardens has been disproved. The plant has successfully colonized the Loire Valley, where it is still found today, along with the Rhone Valley, an important focus of infestation. In France alone, it was estimated in 2020 that there are between 1 and 3.5 million people allergic to ragweed pollen. In Italy, currently in the region of Lombardy and Piedmont ragweed pollen allergy is prominent among respiratory allergies.

The first reports of allergic sensitization to ragweed in Europe have been published since the 1980s. Sensitization rates to ragweed pollen vary widely by country (the highest being reported in Hungary, Northern Italy and France). Today, ragweed pollen allergy is recognized as a public health problem, correlated with the high level of pollen load in the ragweed season, the allergenicity of the pollen, and the increasingly common allergic sensitization in exposed populations. In Eastern Europe, including Romania, ragweed is an important allergenic source, with potential impact on health. Through aerobiological studies from the beginning of the 2000s, the problem of massive ragweed infestation of Pannonian Plain neighboring countries was evaluated, the presence of the plant being associated with a high pollen load during the flowering season.

In 2013, through a study addressed to allergists in Romania, it was documented that both the high morbidity values associated with exposure to ragweed pollen and the severity of the respiratory symptoms specific to the allergic disease induced by this outdoor allergen are recognized. A total of approximately 6 million Romanians are exposed to ragweed pollen, in the West, but also in the South, the rest of the regions being less affected. The prevalence of ragweed allergy was estimated at 5.35% of the active population of Romania, from European data. The European Commission estimates that in Romania, out of the active population (of 9,000,000 people), 482,000 people are allergic to ragweed pollen (Bullock J et al, Ragweed Final Report, European Commission, 2012). These data indicate a prevalence of ragweed allergy of 5.35% in the working population in Romania, corresponding to an increased morbidity (comparable to that for diabetes). Also, recent studies have shown that patients with a disease history of more than 10 years develop severe asthma during the ragweed pollen season, with a strong negative impact on the quality of life (Panaitescu et al, EAACI Online Repository 2015).

In Timișoara, between 2007-2010, the project PREVALERG/PN II 41-011 (http://prevalerg.umft.ro), coordinated by Prof. Dr. Carmen Panaitescu (Bunu), took place. The

project represented the first large-scale research in Romania regarding the evaluation of the impact of aeroallergens on health. The aim of the project was to evaluate the correlation between the allergic status of patients with asthma and/or rhinitis and the type and degree of atmospheric load of aeroallergens. The impact of exposure to respiratory allergens on asthma patients was evaluated, highlighting the importance of exposures to pollens and house dust mites for the persistence of allergic diseases. In the case of ragweed pollen, allergic sensitization has been shown to be present in over 60% of the allergic population, including children. It was also highlighted that ragweed pollen is the most important outdoor allergen.

The INSPIRED project (Innovative strategies for the prevention, diagnosis and therapy of respiratory diseases induced by ragweed pollen), which also includes the research described in this paper, was proposed aiming to continue the line opened by previous studies in the field in Timișoara. The project was implemented in the period 2016-2019 (with an extension of one year) at the Oncogen Center, within the structure of the Timișoara County Emergency Clinical Hospital, where the experimental part was carried out. The main objective of the project is to determine the sensitization patterns of patients allergic to ragweed pollen, in order to support the further development of innovative strategies for the prevention, diagnosis and therapy of ragweed allergy. By characterizing ragweed allergens in detail and the immune response of allergic patients, a diagnostic kit based on recombinant allergens can be developed. This kit, based on multiplex technology, allows molecular diagnosis of allergy and better targeting of disease therapy. The kit can include the major allergen Amb a 11, recently discovered and difficult to produce in recombinant form, under conditions where a significant degree of IgE sensitization is proven, as well as the clinical relevance of this sensitization. Also, through the animal model of immunotherapy with recombinant allergens, the immune response to them can be evaluated, with the generation of IgG type antibodies. They, in turn, can be used for environmental detection of allergens from ragweed pollen.

During a collaboration with a private company (Biomay AG, Vienna, Austria), initiated in 2013, it was revealed that not all patients with severe symptoms and a positive skin prick test to ragweed extract also have elevated serum levels of specific IgE to Amb a 1, 10% being Amb a 1-negative despite the characteristic symptomatology and positive skin tests to ragweed pollen extract. This justifies the need to identify the entire panel of potentially allergenic fragments from ragweed pollen (Panaitescu et al, Allergy 2014), hence the originality mark of the doctoral thesis and its usefulness in fundamental research, as well as the possibilities of subsequent application. The expected results for the doctoral study included the production of the allergen Amb a 11 in the recombinant form, as a stable protein in aqueous solution, and showing an immunogenic character. This study could have a positive impact in the diagnosis of ragweed allergy by immunologically characterizing the major allergen Amb a 11 in ragweed pollen and determining its clinical relevance, especially from the perspective of association with asthma. As a result of this study, the prevalence of IgE sensitization to Amb a 11 in the studied population will also be determined. The data obtained may have a future role in the personalized therapy of ragweed allergy, through allergen-specific immunotherapy using hypoallergenic products.

CURRENT KNOWLEDGE

The general part includes three chapters taking a deeper view on the problem of allergies as a hyperimmune pathology with the pathophysiological mechanisms involved, ragweed pollen as a source of allergens with a brief description of major and minor allergens, as well as the utility of recombinant allergens in the diagnosis and therapy of allergic disease.

The first chapter places allergy in the wider context of IgE-mediated conditions, as well as in the clinical framework created by the association of allergic disease with anaphylaxis, hyperIgE syndromes and atopic status. A description of the pathophysiological mechanisms of type I or immediate hypersensitivity follows, with its characteristic element - the presence of allergen-specific IgE antibodies fixed on the surface of effector cells - mast cells and basophils. The stages of allergic sensitization are described, with the crossing of the allergen through the skin-mucosal barrier, the aggression on the epithelium, the capture of the allergen and the antigen presentation by antigen-presenting cells, together with the development of immune memory. The immediate and delayed allergic response are further developed and their mechanisms are described. Chronic allergic-type inflammation with associated changes are also presented. Immunotolerance is described both as a physiological mechanism by which the development of an allergic-type immune response is sometimes successfully limited, and as immune tolerance induced by immunotherapy, with the genesis of a tolerogenic environment, with allergen-specific IgG and the proliferation of B lymphocyte clones and T regulators.

The second chapter addresses the issue of ragweed and ragweed pollen allergens. In the pollen season, ragweed pollen is well represented among airborne particles designated as biological pollutants. Pollen dispersion models help to predict its distribution in the atmosphere, but to be as accurate as possible, they require input of data with pollen level measurements in as many stations as possible. Unfortunately, pollen monitoring stations are almost absent in Romania. The structure of the pollen grain is then briefly described, with the protein type of allergenic components. These components have been identified beginning in the 1960s, through proteomics techniques. Major allergens are represented by Amb a 1, the best characterized, respectively Amb a 11, discovered in 2013 and characterized since 2015. Minor allergens include members of protein families called panallergens, which comprise proteins with a high degree of AA sequence homology, thus having similar structure and functions. Some minor allergens from ragweed pollen are part of the profilin family (Amb a 8), polcalcins (Amb a 9 and Amb a 10) or non-specific lipid transfer proteins nsLTP (Amb a 6). The other allergens have been less studied and characterized, with the exception of Amb a 4 - defensin, with an Art v 1-homologous structure.

The third chapter deals with the diagnostic and therapeutic approach to allergic disease from the perspective of using recombinant allergens. The usefulness of allergenic extracts in diagnosis and therapy is limited by factors related to the source from which they are processed, the extraction methods and the uncertain composition in molecules with allergenic potential. "Molecular extracts", composed of recombinant allergens, would offer advantages that combine the advantages of using extracts from natural sources (diversity of allergenic molecules, easy applicability, the possibility of dual use - in diagnosis but also as an immunotherapeutic product) with those of using molecular allergens (characterization precise identification of allergens, stability, identification of clinical consequences of sensitization to individual molecules). It is then described how to obtain recombinant allergens step by step, in prokaryotic or eukaryotic expression systems, and the possibilities of using

these allergens. The molecular diagnosis of allergy benefits greatly from the use of recombinant allergens, through the detection of allergen-specific IgE in the patients' serum. The ImmunoCAP™ singleplex test is the Gold standard for in vitro diagnostics, with available tests for more than 100 molecular components, respectively over 400 allergenic sources. In the case of multiplex diagnosis, the gold standard is the ISAC technique. In its current version, it has 112 molecular allergens measured in triplicate on a chip, of which 45 are natural allergens and 67 are recombinant. Allergen-specific immunotherapy is the disease-modifying treatment in allergy, through mechanisms associated with the induction of immunotolerance: production of allergen-specific IgG1/IgG4, induction and stimulation of regulatory T and B lymphocytes, promotion of a tolerogenic environment at the expense of type 2 cytokines. The immunotherapeutic formulation must be immunogenic but bind preformed IgE as weakly as possible in the allergic patient, while also inducing as little de novo IgE synthesis as possible. The occurrence of late cellular reactions, a potential complication of T-lymphocyte stimulation, must also be avoided. To avoid IgE binding, the following have been proposed: the use of fusion proteins, the modification of the amino acid sequence with the creation of hypoallergenic proteins (with editing of the sites recognized by IgE) , the use of fragments of allergenic molecules that do not bind IgE (which are not part of the epitopes recognized by IgE), the use of native or recombinant allergens in the "unfolded" form with disturbed tertiary structure, the polymerization of allergenic molecules or the use of short peptides - epitopes for lymphocytes T. All these hypoallergenic proteins can be produced as recombinant, structurally modified allergens.

PERSONAL CONTRIBUTIONS

The study group was represented by 279 patients, recruited prospectively in the period 2017-2020. Inclusion criteria: adult age (over 18 years), positive status for ragweed pollen allergy confirmed by skin test (SPT) and/or positive ragweed-specific serum IgE, no history of AIT or oral corticosteroid therapy in the last month. Medical procedures (SPT, peripheral venous blood collection) were performed after obtaining informed consent. Prior to the start of the study, approval no. 24/2017 of the Scientific Research Ethics Commission, Victor Babeș University of Medicine and Pharmacy in Timișoara (UMFVBT), and opinion no. 102/2017 of the Local Ethics Committee for Scientific Research of the Timișoara County Emergency Clinical Hospital, were obtained For the experimental animal study, two New Zealand White rabbits were used for immunization with each recombinant allergen. The animals were housed for the duration of the experimental study (2018/2019) in the "Horia Cernescu" Research Laboratory Complex of the University of Agricultural Sciences of Banat King Mihai I of Romania, Timișoara and later were given up for adoption. Prior to the start of the study, approval no. 24/2017 of the Scientific Research Ethics Commission, UMFVBT, Project Authorization no. 001/2017 of the Timiș Sanitary-Veterinary and Food Safety Directorate.

The following methods were employed:

- bibliographic research: study of references regarding the pollen season in countries neigboring the Pannonian Plain, pollen distribution maps
- in silico methods: prediction of physico-chemical parameters, Amb a 11 homology analysis, protein structure modeling

- statistical methods for data processing (primary statistics, comparison tests, risk analysis, incidence and prevalence)
- in vitro experimental methods: expression of recombinant allergens in a prokaryotic (E. coli) or eukaryotic (Spodoptera frugiperda cell line Sf9) expression system, allergen purification by affinity chromatography, detection by polyacrylamide gel electrophoresis (SDS-PAGE), physicochemical characterization by MALDI-TOF mass spectrometry and circular dichroism analysis, Western blot for His-tag detection and for the detection of recombinant allergens IgE binding in the serum of allergic patients, the immunoenzymatic method (ELISA) for measuring the frequency of IgE binding of recombinant allergens for the whole study group, humanized rat basophils (hRBL) degranulation assay, recombinant allergen custom ImmunoCAP testing
- in vivo experimental methods: immunization of laboratory animals (rabbits) with recombinant allergens, followed by evaluation of the immunogenicity of these allergens

RESULTS AND DISCUSSION

Modeling of pollen distribution in Europe indicated a significant and continuous exposure of the Pannonian Plain inhabitants to ragweed pollen. From the second half of July until the end of October, pollen was detectable in the air, with pollen loads high to very high for much of the season. From the modeling of the spatial distribution of pollen in the atmosphere, a remarkable overlap with the outline of the Pannonian Plain can be observed, throughout the time interval corresponding to the pollen season.

Ragweed pollen has been recognized since the early 2000s as a major biological air pollutant in the Western Plain. Romanian studies from that time already describe ragweed pollen as an important component of atmospheric pollen, it being present in the highest absolute amount (3600 grains/m3, cumulative value for the year 2003), with the pollen season described for the months July-September (after September no more data were presented). In 2004 pollen was again identified, sporadically even in a few days from the end of June, then relatively frequently in July and in maximum quantities in late August-early September.

Based on data available from pollen load modeling for the Pannonian Plain, in conjunction with case incidence data for the study group, it is proposed that for certain areas of the Pannonian Plain, including Timișoara Metropolitan Area, the ragweed pollen season extends for at least 3 months, starting from mid-July until the end of October.

The homology study, showing a low degree of homology between Amb a 11 and other allergens of the cysteine-protease family, indicated the maximum value of structural homology of 40.49% to oil palm polygalacturonase. Only at a degree of homology above 70% are there premises for cross-reactivity, so Amb a 11 is not expected to exhibit cross-reactivity with other allergens of the cysteine-protease family.

In the current study, the IgE immunoreactivity of the two forms of recombinant allergens (eAmb a 11 obtained in E. coli, iAmb a 11 obtained in insect cells) is 50% and 60%, respectively, and the values described in the literature are comparable. Through the ELISA technique, a global IgE immunoreactivity of 63.44% was determined, against at least one of the recombinant allergens (rAmb a 11). A very high degree of statistical correlation was calculated between the OD values obtained by the ELISA technique ($r=0.9$, $p<0.05$), using the two allergens eAmb a 11 and iAmb a 11 immobilized on plates, and the same series of sera .

This is an indication that the specific IgE epitopes are also similar in the case of the two molecules, or at least the immunodominant ones, because the probability of 279 patients having a similar polyclonal IgE immune response pattern is low, if the specific IgE epitopes do not match between those two allergenic molecules. In addition, molecular modeling revealed the eccentric arrangement of the hexahistidine marker with respect to the central area of the protein, with a minimal steric influence on the access of antibodies to the allergen bulk, both in the case of the monomer and in the case of the modeled homodimer.

The recombinant protein was also characterized by measuring the circular dichroism spectrum. The values obtained in the present study indicated a lower percentage of alpha-helix - 14% according to the model used for deconvolution - in the eAmb a 11 (attributable to the more difficult folding of the protein, with 3 disulfide bridges, in the prokaryotic cell). In the iAmb a 11, a secondary structure content with more than 50% alpha helix was determined, possibly due to production in insect cells with a more adequate cellular equipment for folding complex proteins, but also due to glycosylation status that could have a stabilizing effect on the secondary structure of the protein.

Weak bands indicating the presence of low molecular weight proteins were identified by SDS-PAGE. These may occur as a result of the partial, self-catalyzed proteolysis of the allergen (which in its mature form has been identified as a cysteine-protease). Correlating the appearance of the bands in SDS-PAGE with the His-tag detection in Western blot, it is noted that the band immediately above 10 kDa was missing His-tag, suggesting that this protein is from the N-terminus, while the proteins at about 28 and 17 kDa are those containing the C-terminal His-tag of the recombinant allergen. However, these proteolytic breakdown products did not appear IgE-reactive on immunoblot indicating a loss of secondary structure compared to the original allergen, with the lack of epitopes that could be recognized by specific IgE. Bands at approximately 36 and 72 kDa were detected on the immunoblot, which indicates the immunoreactivity of the patients' serum against the intact recombinant allergen, as well as against its dimers. In addition, proteolysis products appear more frequently, in SDS PAGE and hexahistidine marker detection, in the case of eAmb a 11, their quantity and number being lower in the case of iAmb a 11. This phenomenon can be explained by the fact that glycosylation reduces the degree of proteolysis in the case of iAmb a 11 as has been reported in the case of other allergens with protease activity.

The biological effect exerted by rAmb a 11 on humanized rat basophils is equated with the release of the inflammatory mediator β-hexosaminidase, as a result of basophil degranulation. The mediator release described in the present study was generally weaker than that induced by nAmb a 1, measured over a wide range of allergen concentrations used (from 10 μg/mL to 1 pg/mL, respectively 0 negative control). However, the recombinant allergens rAmb a 11 induced degranulation, in some cases (patients A, C), of almost the same magnitude as the natural allergen Amb a 1 used as a comparator. Consequently, Amb a 11 shows allergenicity, translated in vivo by degranulation (followed by the appearance of symptoms), at least for some of the patients allergic to ragweed pollen. Degranulation of effector cells is responsible for an important component of the symptomatology of allergic disease. The phenomenon may also be one of the causes of the alteration of quality of life indicators in patients from the Amb a 11 positive subgroup, even if the differences between them and those from the Amb a 11 negative group did not reach the threshold of statistical significance.

In the animal model used in the present study, which can be described as an immunotherapy model, rAmb a 11 allergens were inoculated to obtain rabbit polyclonal IgG

antibodies. The immediate utility is to demonstrate the immunogenicity of the recombinant allergen. Subsequently, the antibodies can be characterized and used in a panel to measure environmental allergens. Hypoallergenic versions of the recombinant protein can also be designed, which are used in the development of allergen-specific immunotherapy, on a molecular basis. All four rabbits, two for each of the recombinant forms of Amb a 11, produced IgG in detectable titer by ELISA even at 1:10000 dilution of serum. The antibody titer, equivalent to the OD in ELISA detection, was higher in the case of iAmb a 11, which may indicate a higher immunogenicity of the glycosylated form of the recombinant allergen. While similar antibody titration curves were induced by eAmb a 11 in the two rabbits, in the case of those for iAmb a 11 a different response is noted between the other two rabbits.

Looking at the clinical data, the vast majority of patients (over 70%), both male and female, were young adults under the age of 40. Men were overrepresented in the 18-39 age group ($p<0.05$). The average duration of the allergic disease, recognized by the patients (n=279), was 5.17 years, with great heterogeneity (including 22 newly diagnosed cases, 7.9% of the total). The average age of ragweed pollen allergic patients was 35 years. It is almost unanimously recognized that allergic disease favours young people, even more so when it overlaps with atopy, and the atopic march begins in early childhood. In the USA, NHANES III (The Third National Health and Nutrition Examination Survey) was conducted between 1988 and 1994. This is a cohort study of 12585 people, in which allergic status was also assessed by SPT. The results indicate different prevalence of allergies according to age, sex, race/ethnicity as well as the geographical area where the subjects originate. The 2005-2006 NHANES study was conducted with 10,348 participants. A higher IgE seroprevalence to aeroallergens was reported in males ($p<0.05$).

The mean nasal symptom score of 7.83 (median 8) on a scale of 0 to 12 indicates that most patients had severe symptoms. These were: nasal obstruction, rhinorrhea, sneezing and nasal itching, recognized as cardinal symptoms of allergic rhinitis and present in numerous patient evaluation scores such as TNSS, RQLQ. The scoring system adopted in the present study is similar to the TNSS, each symptom being quantified on a scale from 0 to 3. In the Romanian study (Bocșan et al.), an average TNSS of 5.76 and 9.21 was determined for patients allergic to ragweed pollen from the two centers. The mean conjunctival symptom score was 4.7 (median 5), on a scale from 0 to 9, indicating that most patients had moderate-severe symptoms. This score adopted in the present study is similar to the TOSS score, with symptoms quantified on a scale from 0 to 3: lacrimation, ocular itching, conjunctival inflammation (total 0-9 points). In a study published in 2021, the exposure in the pollen room of presumably allergic patients to birch pollen, respectively the conjunctival challenge with this pollen, was investigated. The TOSS value ≥ 5 was considered as the threshold for a positive response. At the second exposure, 75% of the patients responded positively, a percentage remarkably similar to the prevalence of allergic conjunctivitis in the Romanian studies.

In the current study, most patients were polysensitized (198 cases, 70.96%). The most frequent associations described: allergic sensitization to cereal/grass pollen (51%), then to house dust mites (47%) and to mugwort pollen (37%). The prevalence of positive SPT results in the NHANES III population study was assessed at 54.3% for at least one positive test, with an almost equal prevalence (26-27%) being identified for positive testing in house dust mites, rye pollen, ragweed and German cockroach (Blatella), then 17% for cat, 12.9% for Alternaria among fungi. The following risk factors for a positive test were identified: age between 20 and 29 years, male sex, minority race, resident of the Western states, living in old housing, and low serum cotinine level.

The quality of life parameters monitored in the present research are sleep disturbances, the presence of hospitalizations (including emergencies) due to allergy and the global activity impairment score. Almost half of the women reported sleep disturbances of moderate intensity, while more than a third of the men reported no sleep disturbances. Also, hospitalizations due to allergy are twice as frequent in the case of women, and the average global activity impairment score is one unit higher. All these differences regarding the quality of life impairment in women compared to men are statistically significant ($p<0.05$ S). A 2022 study explored data obtained in the 2005-2006 NHANES from the perspective of QoL impairment in allergy using logistic regression models. A number of 2648 well-characterized patients were selected from the perspective of allergies, sleep disorders and covariates included in the regression models (obesity, diabetes, cardiovascular disease, alcohol consumption, smoking, etc.). A strong association of sleep disorders with allergic diseases has been demonstrated: reduced sleep duration, obstructive sleep apnea, daytime sleepiness. Insufficient sleep duration has been associated with the occurrence of allergic sensitization. Also, sleep disturbances, obstructive sleep apnea, and daytime sleepiness were reported more frequently and statistically significantly for women.

The logistic regression model in the present study was performed to explore the discrimination, based on covariate variables, between severe asthma-like symptoms (symptom score$\geq$3) and less severe symptoms. Given the possible underdiagnosis of asthma in the population, as well as the reduced accessibility to spirometry services, formal asthma diagnosis was not a strict criterion. After running the model, several variables were identified that successfully discriminate between more severe and less severe respiratory symptoms: IgE sensitivity to Amb a 11 (OR = 4.82, 95% CI = 1.81-12.81), SPT positive result for dog dander, domestic exposure to cat. The difference between the two allergenic sources from pets seems to indicate that dog exposure would not be a risk factor with severe respiratory symptoms unless IgE immunoreactivity is manifest (association demonstrated by OR = 7.48, 95% CI = 2.29 - 24.46 for SPT positive result to dog dander), i.e. the natural immunotolerance has been overcome. In contrast, cat exposure (in the past 12 months or more) would be sufficient to be associated with the risk of more severe respiratory symptoms (OR = 3.3427, 95% CI = 1.29 - 8.65, for cat exposure in indoor environment).

The association between cat exposure and asthma-like symptoms was also described in a previous study with cat-allergic children, the exposure being sufficient to be at risk of allergic symptoms such as wheezing or nasal symptoms. In another pediatric study (n=300), the influence of Der p 1 concentration in the environment (cysteine-protease specific to house dust mites) on the severity of asthmatic symptoms was investigated. Other variables (environmental load of Der f 1, Fel d 1, Can f 1 and Bla g 1) were also included in the regression model. A 5-level asthma severity score based on the GINA 2002 guideline (graded from 0, 1 - intermittent, to 4 - severe persistent) was used. A risk of higher asthma severity level was demonstrated for children exposed to concentrations above 2 µg Der p 1 /g house dust. Also, the combination of sensitization (presence of allergen-specific serum IgE) with environmental detectable exposure to Can f 1 and Fel d 1 was associated with greater asthma severity (OR = 2.06, 95% CI 1.01, 4.22 for the first allergen, respectively OR = 2.41 95%CI 1.19, 4.89 for the second).

All other variables included in the logistic regression model in the current study were removed by the regression algorithm when they reached the predetermined threshold of $p>0.25$. The value obtained for AUC=0.743 is considered acceptable level (0.7-0.8) for the ability of the test (using the identified variables) to discriminate between positive results (asthma-like symptom score greater than or equal to 3) and negative ones .

CONCLUSIONS

The study described in the doctoral thesis is the first worldwide work achieving production and characterization of the major allergen Amb a 11 as a mature protein. I obtained the allergen in recombinant form, in two different expression systems and in parallel. The aims I pursued were experimental - the characterization of the allergen in recombinant form with the determination of its allergenic and immunogenic activity, epidemiological - the assessment of the prevalence of sensitization to this allergen in a defined population and risk analysis, as well as clinical - the investigation of the clinical significance of IgE immunoreactivity to Amb a 11, characterization of allergic patients. Thus, the research has an experimental component (performed by molecular allergology methods, animal model experiment) and a clinical component (performed by a study with prospectively recruited patients and cross-sectionally analyzed clinical parameters). In the light of the data presented, the following conclusions can be expressed:

1) Under favorable climatic conditions, the pollen season for ragweed in the Western Plains can extend over 3 months, from mid-July to late October.

2) The incidence of recruited cases of patients allergic to ragweed pollen increased during the pollen season, with peak incidence at the end of August - beginning of September (6/100,000 in the studied sample, respectively 6 times the basal incidence).

3) The prevalence of allergic sensitization to rAmb a 11, determined by the ELISA technique, among patients allergic to ragweed pollen was 63.44%, confirming the definition of Amb a 11 as a major allergen in the studied population.

4) Both the recombinant allergen and its dimers were IgE reactive against the serum of ragweed pollen allergic patients, which attests the allergenicity of rAmb a 11 obtained in both expression systems (eAmb a 11 in Escherichia coli, respectively iAmb a 11 in cells of insects).

5) The structural differences between the two recombinant forms of Amb a 11 produced in different expression systems may account for the differences in allergenicity and immunogenicity of the two allergens.

6) The rAmb a 11 allergens were immunogenic, with both forms inducing allergen-specific serum IgG production in immunized rabbits. The IgG titer was significant, detected by ELISA up to 1:100,000 serum dilution in the case of iAmb a 11 and 1:10,000 in the case of eAmb a 11. The recombinant allergen iAmb a 11 demonstrated a stronger immunogenic character than eAmb a 11.

7) In the studied group (n=279) there was a disproportionate presence of male, young patients (in the age group 18-39 years), compared to the proportion of women ($p < 0.05$ S).

8) The majority of patients (70.96%) were polysensitized ($p<0.05$ S), in their case the frequent associations being sensitization to cereal/grass pollen - outdoor environmental allergen (51.52%), respectively to mites from house dust - indoor environmental allergen (47.98%).

9) The median nasal symptom score was 8 (out of a possible 12 points) indicating that the majority of ragweed pollen allergic patients had severe nasal symptoms attributed to allergic rhinitis.

10) Women allergic to ragweed pollen reported more severe impairment of quality of life ($p<0.05$ S), in terms of sleep disturbances, allergy hospitalizations, and global activity impairment score.

11) In a well-characterized subgroup (n=150), from a molecular point of view, patients classified positive by ELISA for IgE sensitization to Amb a 11 had an associated risk of more severe asthma-like symptoms (score 3+) compared to non-reactive patients of Amb a 11 (OR = 4.82, 95%CI: 1.81-12.82).

12) Other risk associations for severe asthma-like symptoms were positive dog dander SPT (OR = 7.49, 95%CI: 2.29-24.46), respectively domestic exposure to cats (OR = 3.34 , 95%CI: 1.29-8.65).

ORIGINAL CONTRIBUTIONS

The original contributions from the presented doctoral research are tracked on three lines:

- the study of applied molecular allergology for obtaining and characterizing the recombinant forms of Amb a 11 (rAmb a 11)
- the experimental model of immunotherapy on rabbits
- the cross-sectional study of prevalence and characterization of allergic patients

1) In the first research component I accomplished the following
   - production of the mature Amb a 11 allergen in recombinant form by both expression systems, in quantities suitable for further molecular allergology applications (the quantitative requirement for recombinant allergens further use)
   - highlighting the immunoreactivity of rAmb a 11 against sera from ragweed allergic patients (the qualitative requirement)
   - characterization of recombinant allergens through molecular modeling and laboratory analysis, necessary for the optimization of molecular allergology techniques
   - evaluation of rAmb a 11 allergenicity by the RBL basophil degranulation test, the degree of allergenicity being a predictor for patient-centered applications feasibility
2) In the study on laboratory animals I demonstrated the immunogenicity of rAmb a 11, attested by the presence of IgG in the serum of rabbits immunized with the recombinant allergens, in sufficient titer for subsequent applications of molecular allergology.

The results of the molecular allergy study and the immunotherapy experimental model show that rAmb a 11 can be used in a panel of ragweed pollen allergens with applicability in the molecular diagnosis of allergy, but also as a starting point for the development of new molecules (peptides , hypoallergenic) for inducing tolerance through immunotherapy. Also, IgG antibodies obtained by immunization of rabbits can be the basis of biosensors for the detection of allergens in the environment. Thus these contributions have an applicative character.

3) Through the clinical component of the research, I achieved the following theoretical contributions:
   - Confirmation of the role of Amb a 11 as a major allergen, with over 50% seroprevalence of allergen-specific IgE antibodies among ragweed pollen allergic patients
   - Characterization of the group of allergic patients, with the following features: the predominance of young male patients, the more severe impact on the quality of life in the case of women allergic to ragweed pollen, the association of allergic sensitization to Amb a 11 with the risk of more severe asthma-like symptoms

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Fundamental research problems remaining unsolved in the field, which can constitute directions for further research: determination of long-term stability and under various environmental conditions of recombinant allergens, identification of IgE-specific and IgG-specific epitopes, design of peptides based on recombinant allergens - for diagnosis and therapy, design of hypoallergenic molecules for allergen-specific immunotherapy.