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THE IMPORTANCE OF CROSS-REACTIVITY IN GRASS POLLEN ALLERGY

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Abstract – According to the data obtained from *in vivo* and *in vitro* testing in Serbia, a significant number of patients have allergic symptoms caused by grass pollen. We examined the protein composition of grass pollens (*Dactylis glomerata*, *Lolium perenne* and *Phleum pratense*) and cross-reactivity in patients allergic to grass pollen from our region. The grass pollen allergen extract was characterized by SDS-PAGE, while cross-reactivity of single grass pollens was revealed by immunoblot analysis. A high degree of cross-reactivity was demonstrated for all three single pollens in the sera of allergic patients compared to the grass pollen extract mixture. Confirmation of the existence of cross-reactivity between different antigenic sources facilitates the use of monovalent vaccines, which are easier to standardize and at the same time prevent further sensitization of patients and reduces adverse reactions.

Key words: allergy; grass pollen; allergens; cross-reactivity; immunotherapy

INTRODUCTION

Grasses constitute a huge family, with around 8 000 species. They are very widespread, probably covering about 20% of the world's surface.

Various conditions are required for a plant to cause pollinosis, such as the capability to distribute pollen by wind, to produce allergenic pollen in sufficient quantities, and its proximity to humans (Vieira, 2002).

Worldwide, at least 40% of allergic patients are sensitized to grass pollen allergens (Freidhoff et al., 1986: Anderson and Lidholm, 2003). Thus, a variety of pollen-producing grasses have been recognized as allergenic, including *Lolium perenne*, *Poa pratensis*, *Phleum pratense*, *Dactylis glomerata* and *Cynodon dactylon* (Weber, 2003). *Lolium perenne* and related grasses are significant sources of allergens in temperate climate regions in North America, Europe and in parts of Australia (Vieira, 2002; Smart et al., 1979; Wüthrich et al., 1995).

Patients with grass pollen allergy, commonly called pollinosis, often manifest reactivity to pollen allergens from a number of grass species due to cross-reactivity of IgE antibodies to pollen proteins present in pollen grasses. An extensive cross-reactivity among the different individual species of the genus could be expected, as well as, to a certain degree, among members of the family *Poaceae*, most likely in members of the subfamily *Pooideae* (*Dactylis glomerata*, *Festuca elatior*, *Phleum pretense*, *Lolium perenne*, *Poa pratensis*, *Alopecurus pratensis*, *Agrostis stolonifera*), (Wüthrich et al., 1995; Anderson and Lidholm, 2003).

Pollen allergens produce clinical symptoms after contact with the airway mucosa and the conjunctiva of previously sensitized individuals. Pollen allergen sensitization may occur in isolation or associated with sensitization to other perennial allergens, such as household dust mite allergens (*Dermatophagoides pteronyssinus,* Dpt).

Developments in studies on allergen sensitization and characterization have increased our understanding of the grass pollen allergen sensitization process in sensitive individuals. A very important step in the diagnosis of allergic diseases is *in vivo* skin prick testing, performed usually with crude extracts. These extracts are also used for specific immunotherapy with allergens, although in the case of grass pollen, allergen potency may vary according to the environmental plant cultivation conditions in species of the same subfamily, the degree of maturity of pollen grains, extraction procedure and extract stability (Niederberger at al, 1998). There is a trend among allergen extract companies to diminish the number of individual extracts available for commercial use (Weber, 2008; Mothes et al., 2004).

Cross-reactivity can be explained in reference to biological taxonomy. The premise is that closely related plants will have a greater number of shared antigens than distantly related ones.

Cloning of recombinant allergens has helped in the identification and confirmation of homologous proteins and has clarified their functions. It has been suggested that protein content, or molecular classification, is a superior way to address cross-reactivity issues rather than botanical taxonomy (Mothes et al., 2004). Even though members of profilins and other major classes of grass pollens allergens contain high amino acid sequence homology, they do not always show strong cross-reactivity.

Thirteen grass pollen allergen groups have been described as relevant antigens. Clinically, group 1 allergens are the most important, and are recognized by approximately 95% of grass pollen sensitive patients, followed by group 5 allergens, which are recognized by up to 85% of these patients (Weber, 2003). Other clinically relevant allergens are those of groups 2, 3, 4 and 13, which are recognized by over 50% of grass pollen-allergic individuals (Fahlbusch et al., 1998). When choosing allergens for immunotherapy and *in vivo* diagnosis, one should be guided by regional factors such as the prevalence of vegetation and climate. *Dactylis glomerata*, *Phleum pretense* and *Lolium perenne* are a frequent cause of allergies in our region. According to the data obtained from *in vivo* and *in vitro* testing in Serbia, a significant number of patients have allergic symptoms caused by grass pollen (Burazer et al., 2004), and most of them are subjected to grass pollen immunotherapy.

The aim of this study is to investigate the composition of the grass pollen protein extracts from our area and the existence of cross-reactivity in patients allergic to grass pollens, in order to optimize *in vivo* diagnosis and specific immunotherapy.

MATERIALS AND METHODS

Skin prick testing

Skin prick testing was performed with a standard battery of glycerinated extracts (grass, tree and weed pollen, home dust, bed-linen dust, animal hair, mold, bacteria, bee venom, wasp venom, hornet venom, cockroach, and single pollens: *Dactylis glomerata*, *Phleum pretense* and *Lolium perenne*, all from the Institute for Virology, Vaccines and Sera "Torlak", Belgrade, Serbia). Histamine phosphate at 1 mg/mL and PBS were used as positive and negative control, respectively. The results of skin prick tests were evaluated after 20 min and a wheal of at least 3 × 3 mm was considered positive.

Specific IgE (sIgE) detection

Specific IgE (sIgE) detection of patients' sera was performed by ImmunoCAP-100 System analysis (Thermo Fisher Scientific, Uppsala, Sweden). The results were expressed in kUA/L and as CAP scores from class 0 to 6, according to the manufacturer's instructions.

SDS-PAGE and Western blot

SDS-PAGE was carried out according to Laemmli (1970) using a SE 600 Ruby scientific instrumentation apparatus with a discontinuous buffer system. Samples were boiled for 5 min before the run. About 16 μg/well of proteins was resolved on a 13% gel at 80 V for 1 h and 250 V for another 2 h. Either the gel was stained with Coomassie Brilliant Blue R-250 (CBB) to visualize the separated proteins or resolved components were blotted by a semi-dry electrotransfer onto the nitrocellulose membrane (0.45 μm, Serva, Heidelberg, Germany). The membrane was blocked in 20 mM Tris-buffered saline (TBS) containing 1 % BSA and 0.1 % Tween 20 for 1 h and dried until development.

IgE detection

IgE-binding proteins were detected by Western blot analysis with 5-fold diluted individual patient's sera, or sera from non-allergic individuals in PBS containing 0.01% v/v Tween 20 (TPBS) with 0.1 % BSA. Alkaline phosphatase-labeled monoclonal anti-human IgE (Sigma Chemical Co., St Louis MO, USA) was used as the secondary antibody. The binding patterns were visualized with a substrate solution of 1.5 mg BCIP (5-bromo-4-chloro-3-indolyl phosphate, Serva, Heidelberg, Germany) and 3 mg NBT (nitro blue tetrazolium, Serva, Heidelberg, Germany) in 10 mL of 100 mM Tris buffer, containing 150 mM NaCl, and 5 mM $MgCl_2$, pH 9.6 according to Harlow & Lane (Harlow and Lane, 1990).

RESULTS

The data obtained from patients included in immunotherapy in 2010 and 2011 show that grass pollen stands out as the second relevant cause of allergic conditions in Serbia. The major cause of allergy in patients that underwent immunotherapy is household dust mite allergens – Dpt (758/2010 and 830 /2011), compared to the number of affected patients who received immunotherapy for group grass pollen allergy (311/2010 and 298/2011) (Fig. 1).

The number of patients receiving therapy for individual grass pollens (monovalent vaccines) is presented in Fig. 2. The most frequent immunotherapy is for *Dactylis glomerata* grass species.

Qualitative analysis of allergen extracts (*Phleum pretense, Lolium perenne* and *Dactylis glomerata*) used for *in vivo* diagnostics and immunotherapy is presented in Fig. 3. SDS-PAG electrophoresis of samples showed the existence of protein strips ranging from 10 to 116 kDa with the most prominent protein bands on 65, 60, 35, 18 and 10 kDa in all 3 extracts. Those protein bands are marked as major allergens (groups 1, 4, 5, 6, 11 and 13) and are in accordance with the literature data (Morata et al., 2005).

The allergen profile of single pollen extracts was tested by immunoblotting using the sera of patients allergic to a mixture of grass pollen and to individual pollen extracts of *Dactylis glomerata*, *Phleum pretense* and *Lolium perenne.*

Fig. 4 shows the IgE immunoreactivity of each patient's sera (sIgE levels were measured by ImmunoCap test against a mixture of grass pollen extracts) towards proteins from *Dactylis glomerata*, *Phleum pretense* and *Lolium perenne* antigenic extracts. The immunoreactive pattern is almost identical within a patient group for each of the three grass pollen extracts, but differs substantially between *Dactylis glomerata*, *Phleum pretense* and *Lolium perenne* antigenic extracts.

Fig. 5 presents the IgE immunoreactivity of individual patient's sera (sIgE levels were measured by ImmunoCap test against individual grass pollen extracts) towards proteins from *Dactylis glomerata*, *Phleum pretense* and *Lolium perenne* antigenic extracts. Fig. 5 shows the immunoblots of patients allergic to a single grass pollen species (as shown by ImmunoCap test) against the SDS-PAGE pattern of individual grass pollen extracts (e.g. serum of patient allergic to *Dactylis glomerata* pollen extract was blotted against same extract and the same stands for other two extracts). Fig. 6 clearly demonstrates what could be anticipated from Fig. 4, which is cross re-

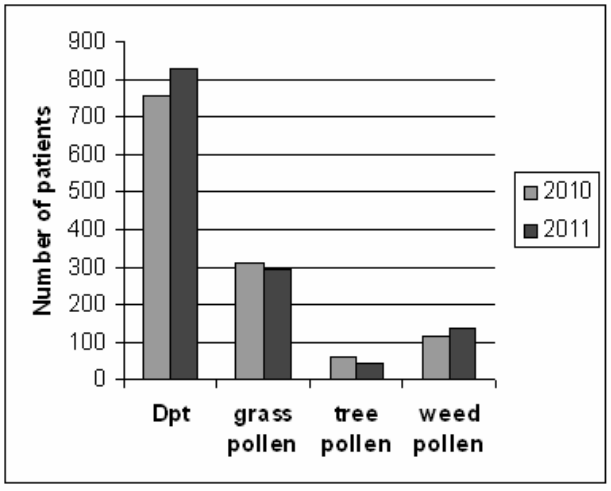

Fig. 1. The number of patients receiving immunotherapy according to the allergy causative agents (2010/2011).

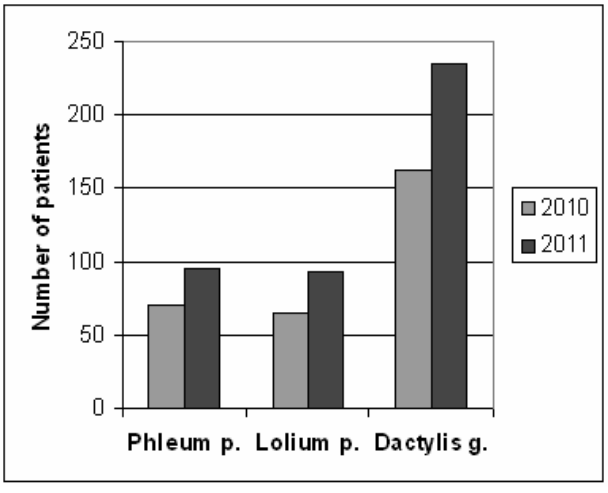

Fig. 2. The number of patients receiving monovalent vaccines for grass pollens (2010/2011).

activity between *Phleum pretense* and each of aforementioned extracts, namely *Dactylis glomerata and Lolium perenne.*

DISCUSSION

The presence of grass pollens, as one of the prevailing causes of allergy, varies worldwide depending on the climate zone. It has been proposed that, due in part to the varied species geography, exposure conditions and patient diversity, no single immunotherapeutic

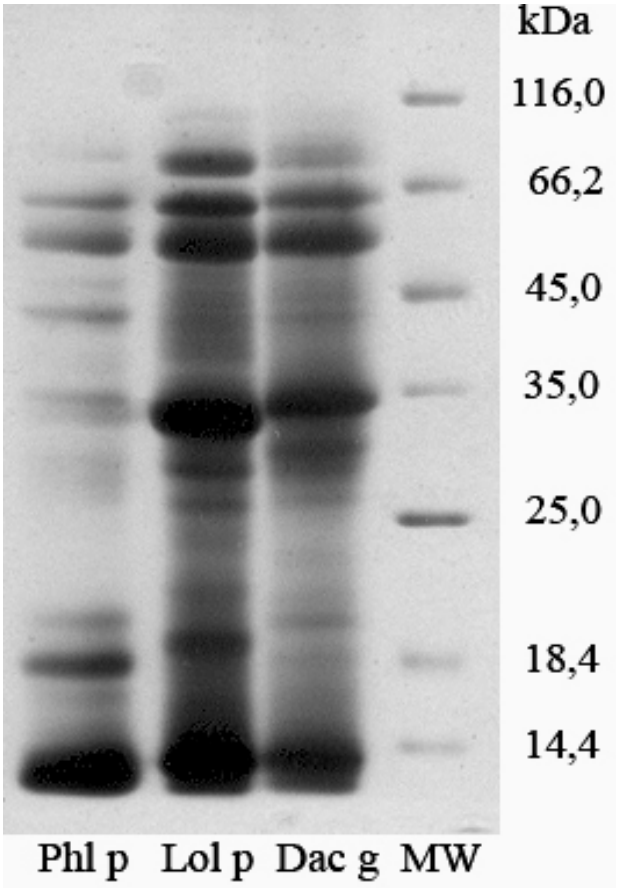

Fig. 3. Separation of pollen extracts - *Phleum pratense, Lolium perenne* and *Dactylis glomerata* by SDS-PAGE on a 13% polyacrylamide gel. MW – molecular mass markers.

approach can be successful in every patients. The most important group of grass pollens is from the *Pooideae* grass species, among which there is cross-reactivity. Traditionally, grass pollen-allergic subjects have been treated with extracts of a combination of grass pollen extracts from different species, which contain large numbers of allergenic proteins.

Grass pollen allergens may present shared epitopes that are responsible for cross-reactivity. Thirteen groups of pollen are described, of which the most important are 1, 5, 3, 4, 2 and 13. Clinically, group 1 allergens are the most important, and are recognized by approximately 95% of grass pollen sensitive patients, followed by group 5 allergens, which are recognized by up to 85% of these patients

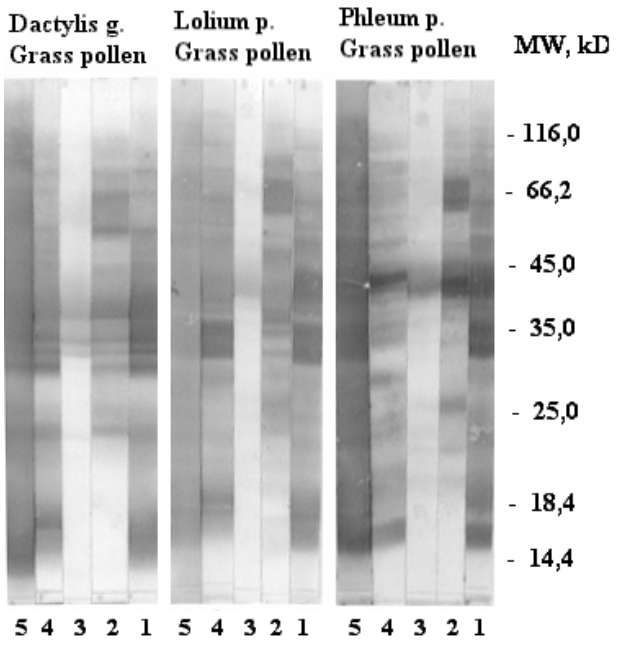

Fig. 4. IgE reactivity from patients' sera allergic to a mix of grass pollen extracts and blotted against individual *Dactylis glomerata, Lolium perenne* and *Phleum pratense* protein extracts. MW – molecular mass markers.

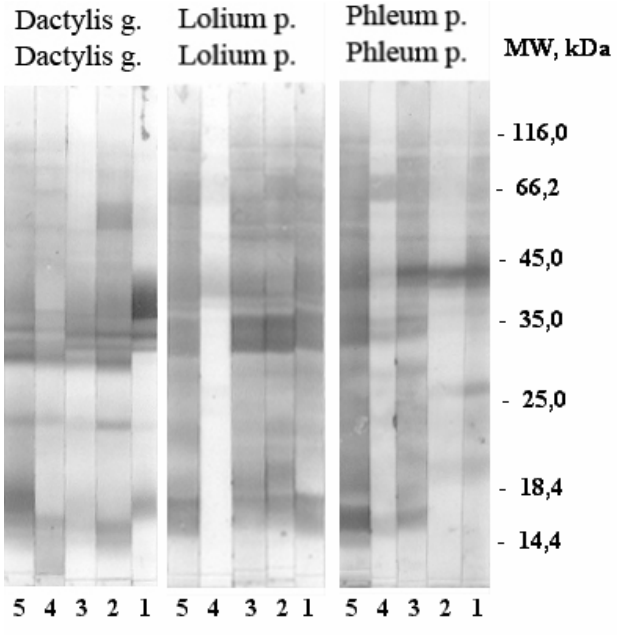

Fig. 5. IgE reactivity from patients' sera allergic to individual grass pollen extracts and blotted against its corresponding pollen protein extract from *Dactylis glomerata, Lolium perenne* and *Phleum pratense.* MW – molecular mass markers.

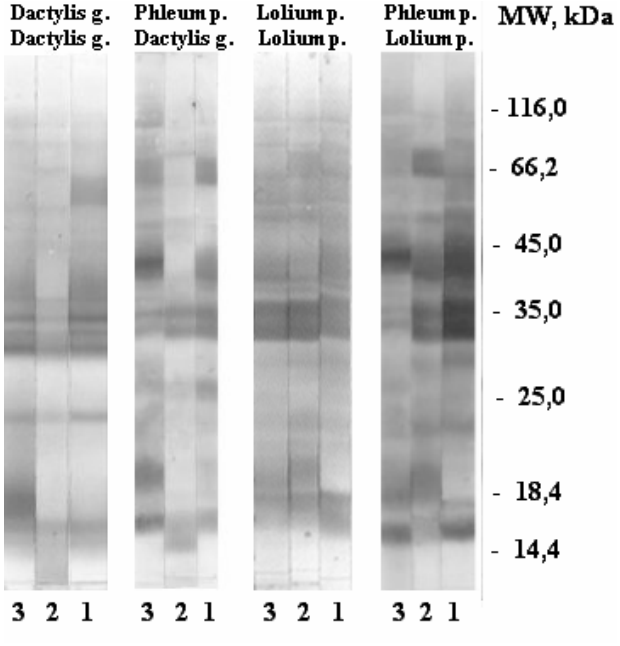

Fig. 6. IgE reactivity from patients' sera allergic to individual grass pollen extracts (*Dactylis glomerata, Lolium perenne)* towards corresponding pollen protein extracts from *Dactylis glomerata, Lolium perenne* and *Phleum pratense*. MW – molecular mass markers.

(Weber, 2003). Other clinically relevant allergens are those of groups 2, 3, 4 and 13, which are recognized by over 50% of grass pollen allergic individuals (Fahlbusch et al., 1998).

Specific immunotherapy (SIT) is recognized, aside from allergen avoidance, as the only treatment "that may affect the natural course of allergic diseases" (Bousquet, 1998). This treatment refers to IgE-mediated allergic diseases. Sublingual immunotherapy (SLIT) for grass pollen-induced rhinoconjunctivitis has been developed to make immunotherapy available to a broader group of allergic patients. Many immunotherapies are manufactured from natural raw materials or crude extracts.

The allergen content of crude extracts may vary not only among different grass species and allergen isoforms, but also according to pollen maturity, allergen extraction procedures and extract stability

(Niederberger, 1998). Quantification of the main allergens contained in extracts and characterization of the main grass allergens, including isoforms, are important to develop allergen extracts to optimize the diagnosis and immunotherapy in sensitized patients.

Cross-reactivity between different pollen antigens helps us understand and simplify allergens for *in vivo* diagnosis and immunotherapy. Natural grass pollen allergens exhibit a wide variety of isoforms. Precise characterization of such microheterogeneity is essential to improve diagnosis and design appropriate immunotherapies. Moreover, standardization of allergen vaccine production is a prerequisite for product safety and efficiency.

Our data suggest that there is a high percentage of cross-reactivity between the allergens from *Dactylis glomerata*, *Phleum pretense* and *Lolium perenne* that were tested compared to the mixture of grass pollens and to single pollens using the sera of patients from our region. The data provide us with the possibility of monovalent immunotherapy application instead of the recent practice, based on therapy using different grass-pollen mixtures. There appears to be no significant differences between single species extracts and mixes (Hajl et al 2009). However, cross-reactivity has been confirmed for both IgE and IgG4. IgE is responsible for allergic symptoms, and the shift in balance from a predominant IgE response to one based primarily on other antibody isotypes is intrinsic to the effect of immunotherapy (Larche et al., 2006; Flicker and Valenta, 2003).

Vaccines prepared from a larger number of pollens are difficult to standardize and control as a product. When polyvalent vaccines are used, there is a possibility of additional sensitization of patients due to their exposure to a large number of antigens. The importance of monocomponent vaccines can due to better standardization, significant reduction in adverse reactions and improved immune response. However, therapy should be designed to contain a sufficient amount of major allergens and that treatment may include wide population of sensitized patients.

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