Session d1

MOLECULAR AND CELLULAR ANALYSIS OF POLLEN ALLERGENS.

Olive pollen allergens. New technologies of study

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Type-I allergy is an immunological disorder that is mediated by the production of IgE antibodies against molecules called allergens. The analysis of the allergenic pattern of the biological sources can help to improve the diagnosis and specific immunotherapy of allergic diseases. Pollen from grasses, weeds and trees are sources of allergy, frequently associated to geographical and seasonal conditions. Among them, olive pollen is one of the most important causes of inhalant allergy in Mediterranean countries, where this tree is intensely cultivated. The olive tree belongs to the *Oleaceae* family which includes ash, lilac, privet, forsythia, and jasmine, some of which are relevant species in temperate areas from Central Europe and North America.

Olive pollen extract shows a complex allergogram that includes more than 15 IgE-reactive components. Up date, 10 protein allergens have been isolated and characterized from this pollen (RODRÍGUEZ & col ., 2001; HUECAS & col. 2001; BARRAL & col., 2004). Regarding their prevalence, Ole e 1, Ole e 9 and Ole e 10 have been demonstrated to affect more than 50% of patients allergic to the whole pollen, and therefore, they have been called major allergens. However, many olive allergens have been shown to exhibit a clinical prevalence dependent of the geographical area, probably due to the different level of pollen grains reached in the pollination season within each area. The reported olive allergens display molecular masses from 5.8 (Ole e 6) to 46 kDa (Ole e 9), and there are several ones of around 10 kDa (Ole e 3, Ole e 7, Ole e 10). Most of the olive pollen allergens are acidic polypeptides and show high structural stability. Ole e 3 and Ole e 8 exhibit low sensitivity to thermal denaturation, and Ole e 7 is, in addition, highly resistant to proteolytic attack.

Several olive allergens have been assigned to protein families of known biochemical role, in some cases by the alignment of their amino acid sequences with proteins contained in the Database banks or by their proved functional activity. The primary structures and molecular properties of Ole e 2 and Ole e 7 fit well with those of profilins and ns-LTPs, respectively. Ole e 3 and Ole e 8 are calcium-binding proteins with two and four EF-hand sites, the former of buffer type and the latter of regulatory character. In addition, Ole e 9 has been demonstrated to exhibit 1,3-□-glucanase activity and, therefore, it belongs to the pathogenesis-related type-2 protein family, which is involved in defence mechanisms of vegetable cells. The information on the remaining olive allergens is not enough to ascribe them as members of a specific protein family. Finally, two olive allergens have been found to be glycoproteins: Ole e 1 and Ole e 9; the glycan moiety of the former has been structural e immunological analysed, and it has been demonstrated to contain an IgE-reactive epitope.

cDNAs encoding Ole e 1, Ole e 3, Ole e 6, Ole e 8, and Ole e 10, as well as the two separate domains of Ole e 9 have been expressed in heterologous systems as *Escherichia coli* or *Pichia pastoris*. The recombinant proteins were structurally and immunologically validated and, in several cases, compared with their corresponding natural counterparts. Molecular cloning, DNA sequencing, and recombinant expression, as well as the recently improved techniques for the molecular analysis of proteins such as mass spectrometry, 2D-electrophoretic separation, NMR spectroscopy, have allowed a quick development in the knowledge of the allergenic components of both animal and plant sources.

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A structure-based approach to the development of modified recombinant variants of the major Birch-pollen allergen Bet v 1 for Specific Allergy Vaccination

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Specific Allergy Vaccination (SAV) is an effective and generally well-tolerated treatment of pollen-mediated allergies. Today, the active components in the majority of the vaccines used for SAV against grass- or tree pollen allergies are aqueous extracts of pollen grains collected from the species of grass or tree that cause the allergic reactions in sensitized individuals. In some cases, pollen from other, but phylogenetically related species e.g. alder or hazel in the case of SAV against birch-pollen allergies, may be used for efficacious treatment.

Meticulous standardization of allergen extracts has become a hallmark of the modern extract-based allergy vaccine, and together with clinically optimized regimens a high level of safety, efficacy and reproducibility of SAV has been achieved. However, when extracts of the sensitizing agent itself is used for treatment there is an inherent risk, however small, of eliciting adverse allergic reactions in the patients, that may never be completely eliminated.

The symptoms that allergic patients experience upon exposure to e.g. grass- or tree pollen are triggered by the interaction of certain proteins (allergens) that become released from the pollen grains when inhaled, with allergen-specific antibodies of the IgE subtype present on the surface of effector cells embedded in the mucosa of the upper airways. The interaction between the allergen and the allergen-specific IgE antibodies mediates release of histamine from the effector cells, which, in turn, is the direct cause of the allergic symptoms (rhinitis, blood vessel contraction, shortness of breath etc.). A similar IgE-mediated mechanism is responsible for the adverse events that may be experienced by a patient when a pollen extract is used for SAV in too high a dose. The appearance of allergen-specific antibodies of other isotypes, e.g. IgG, is correlated with SAV and may be beneficial for the allergic patient. Binding of these non-IgE antibodies to the allergen may forestall the formation of complexes between the allergen and effector-cell bound IgE, and hence prevent the occurrence of allergic symptoms.

An active ingredient for SAV which can be used in higher doses for improved efficacy without compromising safety may therefore be modified allergens that are not recognized by the allergic patient's IgE antibody repertoire and therefore unable to mediate release of histamine from the effector cells. The interaction between allergen and antibodies is highly dependent on the allergen's three-dimensional structure, so any treatment of the allergen that compromises the protein's three-dimensional structure would be expected to lead to less IgE-reactive allergen derivatives. When used for SAV, however, any deviation of the allergen derivative's surface from that of the natural protein would also be expected to lead to altered specificities of the non-IgE subtype antibodies that are raised during SAV and such antibodies may be unable to bind - and shield from effector-cell bound IgE - the unmodified natural allergen encountered in nature. Gross distortions of the molecular surface may therefore render an allergen derivative inefficacious in SAV.

We have used a structure-based approach to modify and select recombinant Bet v 1 derivatives with reduced IgE binding and retained capacity to induce antibodies in mice that are able to block the interaction between unmodified natural Bet v 1 and birch-pollen allergic patients' IgE. The basis for this rational mutagenesis approach is a detailed knowledge of the three dimensional structure of Bet v 1 that allows for precise mapping of antibody epitopes and specific mutagenesis of surface exposed amino acid residues.

Genetic variation of Cry j 1 concentration and isoforms in Japanese cedar (*Cryptomeria japonica*, Taxodiaceae)

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Cryptomeria japonica is one of the most important conifer species for timber production in Japan; however, it is a known cause of severe allergic disease. Pollinosis would be reduced by planting *C. japonica* trees

that produce pollen with a low allergen concentration. In addition, the variation in allergen concentration and isoforms among *C. japonica* trees would affect the quality of allergen extract prepared from pollen samples for diagnosis and immunotherapy. The genetic variation in allergen among *C. japonica* trees is an important matter in both clinical aspect and forest management.

We investigated the variation in Cry j 1 concentration, a major allergen in *C. japonica* pollen, among *C. japonica* plus trees, which are superior in fast growth and bole straightness and are used for improved seed and seedling production. The quantification of Cry j 1 was performed by ELISA using monoclonal antibody J1B01 and polyclonal antibodies (anti-Cry j 1 rabbit IgG). We detected up to a tenfold difference in the Cry j 1 concentration among 143 plus trees. This result suggested that the plus trees with low Cry j 1 concentration would contribute to reducing pollinosis.

Four different isoforms with different binding properties to anti-Cry j 1 monoclonal antibodies, J1B01 and J1B07, were identified from three plus trees. One and two of the four isoforms showed low binding affinity to J1B01 and J1B07, respectively, whereas the other had a high binding affinity to both of them. In comparing the cDNA sequences among the four isoforms, it was determined that amino acid replacements at positions 55 and 352 in mature Cry j 1 affected the binding affinities to J1B01 and J1B07, respectively. We developed two Cleaved Amplified Polymorphic Sequence (CAPS) markers associated with the amino acid replacements and designated them as CRYJ1-55 and CRYJ1-352, according to the polymorphic position. The amino acid residue at the targeted position in Cry j 1 produced by each tree can be determined easily using the CAPS markers, without the need for cloning and sequencing.

CRYJ1-55 and CRYJ1-352 segregated in the F_1 population in a Mendelian mode of inheritance, indicating that each of them represented different alleles which belonged to a single locus. Using the CAPS markers, the allele frequencies which represented low binding affinity with J1B01 and J1B07 were estimated as 1.1% and 18.6%, respectively. This result indicated that the frequencies were different among isoforms.

Allergenic composition of the *Parietaria* pollen

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Parietaria pollen allergens are glycoproteins derived from dicotyledonous weeds of the Urticaceae family including *P.judaica, P.officinalis, P.lusitanica, P.cretica and P.mauritanica*. Immunologically, *Parietaria judaica (Pj)* and *P. officinalis* represent the most relevant species since their pollens represent one of the main outdoor sources of allergens in the Mediterranean area able to induce a Type I allergic reaction in almost 50% of all the allergic subjects living in these countries. The composition of the allergenic extracts of the Pj pollen has been extensively studied[1] and by molecular cloning, the two major allergens (Par j 1 and Par j 2)[2,3] have been sequenced and characterised. In addition, inhibition studies have shown that the *Parietaria* pollen contains some other cross-reactive allergens (Ca2+ binding protein and profilin) present in a variety of other pollen plants and food. By means of IgE binding assays, we demonstrated that the use of the recombinant purified Parj2 allergen is able to predict a true sensitisation towards the *Parietaria* pollen meanwhile, the use of the whole crude extract for diagnosis can lead to ambiguous results unable to distinguish between cross-reactivity and co-sensitation [4]. The Par j 1 and Par j 2 allergens are two small polypeptides of 14,400 and 11344 Da respectively belonging to the same family of plant proteins named non-specific lipid transfer protein (ns-LTP). 3D modelling by homology [5] and enzymatic digestion with endoproteinase Lys-C followed by mass spectrometric analysis [6]have shown that both allergens attain a three-dimensional fold consisted with that of this family composed by four alpha helices and a beta sheet. Despite of this structural homology, cross-inhibition experiments have shown that rParj1 and rParj2 allergens have an independent IgE repertoire [3] and represent the two major allergens of this pollen binding most of the human *Pj* specific IgE [2,7].

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Identification of holm oak (*Quercus ilex* L.) pollen allergens by immunoblotting

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Quercus ilex L. (holm oak) is largely diffused in central Italy and in Rome and its surroundings, used in urban gardens and public parks. During the period of its flowering (spring) it produces large amounts of pollen that is spread in the air reaching high concentrations (maximum value of about 700 grains/m^3 of air in 2003 with average concentrations of about 300 grains/m^3 of air during may).

From 1999 to 2002 we carried on an epidemiological survey on people who attend Tor Vergata University, by means of a questionnaire. The analysis of the questionnaires revealed that 4% of the subjects who referred to suffer from allergies stated to have a reaction towards oak pollen. A group of subjects who gave their informed consent and stated to be allergic in the first year interview, was selected and subjected to clinical investigation: complete anamnesis and Skin Prick Test to confirm the auto-diagnosis referred in the questionnaire. 19% of them resulted positive to the Prick test made by the mixture of Fagaceae allergens.

Despite the large amount of pollen produced by oaks and the presence of patients that are allergic to it, little is known of the allergens in oak pollen in general and holm oak pollen in particular.

The aim of this study is to identify the holm oak proteins that can bind IgE of patients who had positive reactivity to the Fagaceae prick test, by immunoblotting and ELISA test.

Materials and Methods: Holm oak pollen was obtained by mature inflorescences from well-characterized trees nearby Tor Vergata University during the maximum period of pollination (may 2002 and 2003). The pollen was ascertained to be pure by light microscopy and stored at -80°C until use. Proteins were extracted in 125 mM ammoniumhydrogencarbonate, 15 mM sodium azide, 1 mM PMSF (1:10 wt/vol) under continuous shaking for 20 hours at 4°C. Particles were removed by centrifugation (12000 g for 30 min at 4°C). The supernatant was lyophilised and stored at -20°C. Before use, extracts were dissolved in distilled water.

The extracts were separated by sodium dodecyl sulphate polyacrilamide gel electrophoresis and transferred onto nitrocellulose membranes. The nitrocellulose membranes were blocked with phosphate-buffered saline 5% bovine serum albumine, incubated with 1:20 dilutions of sera from 4 atopic subjects and probed with peroxidase-labeled antihuman IgE. The IgE binding bands were detected by enzymatic reaction. The 4 patients who gently provided the serum were poliallergic, and have positive reactivity to grasses and tree pollen, such as Fagaceae, Gramineae, *Plantago, Parietaria, Platanus, Betula, Corylus, Olea* pollen.

ELISA tests were also carried on to determine if the IgE bind a proteic or a carbohydrate epitope of the allergens.

Results: Results indicate that IgE of the 4 subjects bound to 5 bands with molecular weights between 113 and 17 kDa. Only a band was recognized by sera of every patients (the 17 kDa band). When the protein extracts were load into the gel without the use of 2-□-mercaptoethanol a band was recognized by the sera of every patient, at about 35 kDa. In this way the proteins runs in reducing condition but the sample buffer lacks of the component that break disulphuric bonds, so the polymers migrate without break their integrity. The 35 kDa protein can be a dimer of the 17 kDa band, which should be the major allergen of holm oak.

ELISA tests showed that IgE of three of the patients bind both proteic and carbohydrate epitopes of the allergenic proteins, while one serum binds only proteic epitopes.

These data suggest that the extractable proteins of holm oak pollen contain more proteins that are potentially allergenic. The 17 kDa band can represent the major allergen of holm oak pollen.
Experiments are in course to purify the major allergen to sequence it, and compare it with other known pollen allergens to clarify its biological role in pollen.

Sequence polymorphism of the major olive pollen allergen (Ole e 1) in defined cultivars

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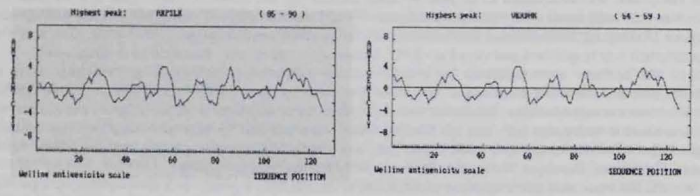
Ole e 1 is considered to be the major allergen from olive pollen. The protein presents several glycosylation forms, and its amino acid sequence displays relevant homology to pollen proteins from maize, tomato, ryegrass, birch, rice, *Arabidopsis* etc. This identity rises to >85% when compared to Ole e 1-like proteins from members of the *Oleaceae* family (lilac, privet, ash, forsythia) (see review of Rodríguez et al., 2002). Ole e 1 itself exhibits microheterogeneities at several positions of its amino acid sequence (Villalba et al., 1994; Lombardero et al., 1994).

In this work, Ole e 1 sequences were amplified by RT-PCR procedures using total RNA from mature pollen of eight different cultivars of olive (*Olea europaea* L.). Ole e 1 amplified sequences were cloned and sequenced. The sequences obtained were submitted to the GenBank™/EMBL Database. The analysis of the obtained sequences showed the existence of a high number of microheterogeneities in the analysed sequences, which were particularly profuse in the 5' and the 3' coding regions. Tree-view analysis of microheterogeneities showed that the inter-cultivar viariability detected was higher than the intra-cultivar variability present in at least three clones of Ole e 1 for each cultivar. An additional N glycosylation motif was detected in one of the cultivars examined. The changes detected within the Ole e 1 molecule affect in many cases immunodominant T-cell epitopes, and produce differences in the hydrophilicity and antigenecity profiles, also affecting the predicted secondary structures of the allergen in the majority of the cultivars studied.

The procedure described here offers a very useful molecular tool to establish discrimination between olive tree cultivars, and to study the basis of the interaction between the allergens and the human immune system. The expression of the obtained clones could be used to define homogeneous Ole e 1 molecules valuable for the improvement of clinical diagnosis and therapy of olive pollen allergy.



Figs. 1 and 2: Antigenicity profiles of the Ole e 1 allergen deduced sequences in two olive cultivars.

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Session d2

CLINICAL ASPECT OF ALLERGENIC POLLEN

Comparison of *Betula* pollen allergens and *Betula* pollen grains concentrations in the air during pollination season 2003 in Cracow (Poland)

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Betula pollen belongs to the most important allergens source and are the main cause of seasonal allergies in Central and Northern Europe. A number of authors have described possibility of releasing the micronic and submicronic allergen-bearing particles from pollen grains especially under moist conditions. These small particles might easy penetrate to the lower respiratory tract and cause symptoms of bronchial asthma. In Cracow volumetric pollen and spores trap monitor its concentration in the air all year round since 1982. In our study we showed that ordinary pollen counts do not reflect concentration of the small particles having antigenic activity in the air.

The concentration of *Betula* air borne allergens was measured on PFDV membrane after capillary blotting from Lanzoni pollen and spores trap tape. For detection and quantification allergens polyclonal antibodies against *Betula* pollen antigens and secondary antibodies conjugated with HRP were used. In the chemiluminescence reaction the light orginating from the antibody-enzyme complex bound to the *Betula* antigens are visualized on x-ray film as black spots. The light was emited at positions in the membrane where antigen was present. The quatification of the allergens was done by the x-ray film densitometry. The resultes of measeurements are given as optical density units (ODu). Obtained results were confronted with palynological data showing pollen grains concentration.

Antigens concentration varied during the whole study period (Marz 10 – May 27, 2003). For this paper the example showing the *Betula* pollen allergens presence whereas no pollen grains was noticed in the air at that time was chosen (Fig. 1.). It might be evoked by relatively high humidity (70 – 90 %) or some unknown conditions caused to attendance of earlier released allergens in the air.

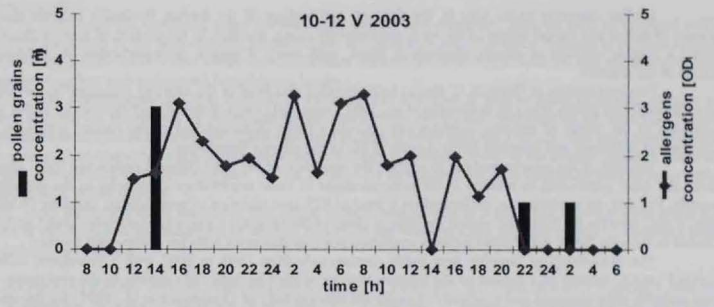

Fig. 1. Comparison of *Betula* pollen allergens and *Betula* pollen grains concentrations in the air on 10-12 of May 2003 in Cracow.