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Therapeutic targets for olive pollen allergy defined by gene markers modulated by Ole e 1-derived peptides

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ABSTRACT

Two regions of Ole e 1, the major olive-pollen allergen, have been characterized as T-cell epitopes, one as immunodominant region (aa91-130) and the other, as mainly recognized by non-allergic subjects (aa10-31). This report tries to characterize the specific relevance of these epitopes in the allergic response to olive pollen by analyzing the secreted cytokines and the gene expression profiles induced after specific stimulation of peripheral blood mononuclear cells (PBMCs). PBMCs from olive pollen-allergic and non-allergic control subjects were stimulated with olive-pollen extract and Ole e 1 dodecapeptides containing relevant T-cell epitopes. Levels of cytokines were measured in cellular supernatants and gene expression was determined by microarrays, on the RNAs extracted from PBMCs. One hundred eighty-nine differential genes (fold change >2 or <−2, $P < 0.05$) were validated by qRT-PCR in a large population.

It was not possible to define a pattern of response according the overall cytokine results but interesting differences were observed, mainly in the regulatory cytokines. Principal component (PCA) gene-expression analysis defined clusters that correlated with the experimental conditions in the group of allergic subjects. Gene expression and functional analyses revealed differential genes and pathways among the experimental conditions. A set of 51 genes (many essential to T-cell tolerance and homeostasis) correlated with the response to aa10-31 of Ole e 1. In conclusion, two peptides derived from Ole e 1 could regulate the immune response in allergic patients, by gene-expression modification of several regulation-related genes. These results open new research ways to the regulation of allergy by *Oleaceae* family members.

1. Introduction

Allergen-specific CD4+ helper T-cell (Th) generation is the initial event leading to the development of allergic disease. Type I allergy is caused by an inappropriate Th2 response to environmental allergens (El Biaze et al., 2003; Schleimer et al., 1989; Walker et al., 1991) leading to antigen-specific IgE production as well as recruitment and activation of proinflammatory cells (e.g., eosinophils and mast cells) in mucosal target organs (Hakansson et al., 1997, 1998; Till et al., 1997). In contrast, under similar exposure conditions, tolerance to allergens is maintained in non-allergic individuals. A deficit in immune tolerance is considered to cause allergy in predisposed individuals. Peripheral T-cell tolerance is characterized by functional inactivation due to specific T-cell subtypes with an immunosuppressive function, generically named regulatory T-cells (Tregs) (Akdis, 2008). IL-10 and TGF-β, secreted by these Tregs, may play an important role (Robinson, 2009; Akdis and Akdis, 2009; Palomares et al., 2010).

Abbreviations: PBMCs, peripheral blood mononuclear cells; qRT-PCR, quantitative real-time polymerase chain reaction; PCA, principal component analysis; Tregs, regulatory T-cells; EAACI, European Academy of Allergology and Clinical Immunology; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

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Olive tree pollen is one of the most important causes of pollinosis in the Mediterranean countries and some parts of Australia and North America. Olive pollen induces mainly nasal and conjunctival symptoms, although it may cause asthma exacerbation in areas with high antigenic load. The major olive allergen, Ole e 1, recognized by around 80% of allergic subjects, is a single polypeptide chain of 145 aa (Villalba et al., 1993; Lauzurica et al., 1988) that shows a high degree of sequence homology and IgE crossreactivity to the main allergens in other *Oleaceae* family pollens such as lilac, privet and, in particular, to ash, a relevant allergen source in areas such as Central Europe and North America. These facts make possible their use as a marker allergen to detect genuine sensitization to this family and in particular to ash (Martín-Orozco et al., 1994; Niederberger et al., 2002; Castro et al., 2007; Lombardero et al., 2002).

Ole e 1 has at least 4 B-cell epitopes (Martín-Orozco et al., 1994) and 2 regions, aa91 to 102 and aa109 to 130 that are defined as immunodominant T-cell epitopes (Cárdaba et al., 1998). Several works have analyzed the IgG and IgE B-cell epitopes of Ole e 1 for developing a molecule-based therapeutic strategy (González et al., 2006; Marazuela et al., 2008; Twaroch et al., 2011).

On the other hand, it was published how continuous exposure to olive pollen lowered the likelihood of patients to be sensitized to olive-pollen allergens (Geller-Bernstein et al., 2002; Florido et al., 1999). Also, a pilot study showed how stimulation with Ole e 1 peptides induced a different cytokine profile in peripheral blood mononuclear cells (PBMCs) from olive pollen-allergic patients compared to non-allergic subjects, according to IL-10 (Cárdaba et al., 2007), being the peptides that included aa10-31 Ole e 1 region, mainly recognized by non-allergic subjects, and postulated as possible immunomodulator peptides. More recently, we have described the decrease of TGF-β sera levels and Foxp3 mRNA expression in olive pollen-allergic subjects, compared with healthy controls and subjects with specific treatment, facts that indicated the lack of regulatory mechanisms in olive pollen-allergic subjects during the pollen season (Aguerri et al., 2012). Further, we have defined a gene-expression pattern that makes it possible to discriminate different clinical conditions related with olive-pollen response in subjects with high exposure (Aguerri et al., 2013).

This report explores how Ole e 1-derived peptides affect gene expression and examines the capacity of these peptides to modulate the levels of Th1/Th2 and Treg cytokines by studying the stimulation of PBMCs of responder and non-responder subjects to this allergen. This study could be important to understand the behavior of Ole e 1 peptides and their capacity to modulate genes and/or pathways that play essential roles in this disease, as well as to establish biomarkers, that might be useful as new therapeutic tools to modulate olive-pollen allergy and allergy to other members of the *Oleaceae* family.

2. Material and methods

2.1. Subjects

The study population comprised 15 untreated olive pollen-allergic patients and 14 non-allergic controls selected from a previous immunological study (Aguerri et al., 2012). The subjects were unrelated and recruited and diagnosed at the Allergy Service of hospitals in Andalusia (Spain), a region in southern Spain with particularly high pollen counts during the pollen season and high prevalence of asthma. Subjects were selected in 2 olive pollen exposure conditions: April to June, with very high pollen counts, and outside the pollen season, October to December, with low pollen counts.

Non-allergic control was composed by subjects with no history of respiratory allergies.

Olive pollen-allergic patients fulfilled the following criteria: seasonal rhinitis and/or asthma from April to June, a positive skin prick test result for *O. europaea* pollen extract (ALK Abelló, Madrid, Spain), and no previous immunotherapy.

All subjects underwent skin prick testing for olive pollen allergy with whole *O. europaea* extract (ALK Abelló) and a battery of common allergens (Aguerri et al., 2012), following the EAACI recommendations (EAACI, 1989).

The exclusion criteria were: current smoking, age outside the correct range, less than 10 years' residence in the study area, and corticosteroid or anti-histaminic treatment.

Informed consent was obtained from each subject. Ethical approval for the study was obtained from the Ethical and Research Committee of the participating hospitals. Biological samples were obtained from Biobanco-FJD, IIS-Fundación Jiménez Díaz Madrid.

Total IgE and *O. europaea* specific IgE antibodies determinations were previously described (Aguerri et al., 2012).

2.2. Olive tree pollen extract and Ole e 1 peptide purification

Olive tree pollen was obtained from Allergom AB, Sweden. The pollen (5% w/v) was extracted with 50 mM ammonium bicarbonate, pH 8.0, containing 1 mM phenyl-methyl-sulfonyl-fluoride, followed by centrifugation at 12 000 × *g* for 20 min at 4 °C. The lyophilized supernatant was stored at −20 °C.

Ole e 1 dodecapeptides were synthesized (purity >90%) according to the Ole e 1 amino-acid sequence (Villalba et al., 1993). The peptides used were: p2 (aa11-22: FHIQGQVYCDTC) and p3 (aa22-33: CRAGFITELSEF) selected for being peptides mainly recognized by non-allergic subjects and inducers of IL-10 (Cárdaba et al., 1998, 2007) and p10 (aa91-102: NEIPTEGWAKPS), p12 (aa109-120: TVNGTTRTVNPL), and p13 (aa119-130: PLGFFKKEALPK) described as T-cell immunodominant regions (Cárdaba et al., 1998).

2.3. PBMC culture

PBMCs were isolated from heparin-containing peripheral blood samples taken during and outside pollen season, by gradient centrifugation on Lymphoprep (Comercial Rafer, Zaragoza, Spain) following the manufacturer's instructions. PBMCs (10^6 cells) were cultured in RPMI 1640 supplemented with 5% FBS inactivated, 1% L-glutamine, 1% peni-streptomycin and 1 mM sodium pyruvate (Flow Laboratories, Irvine,UK) in the absence or presence of olive pollen stimuli: olive pollen extract (*Olea europaea*, 25 μg/ml), peptides 2 + 3 (p2 + 3, 5 μg/ml), peptides 10 + 12 + 13 (p10 + 12 + 13, 5 μg/ml) and PHA (2.5 μg/ml) as positive control, during 24 h 37 °C, 5% CO_2 in humidified air. Concentration and combination of stimuli were based on previous assays (Cárdaba et al., 1998).

After stimulation, the cells were isolated by centrifugation (10 min, 720 g). All the supernatants were used to measure soluble cytokine levels. Total RNA of 6 patients from each group and pollen-exposure moment was extracted using Trizol method (Invitrogen, Carlsbad, CA). A total of 96 RNA samples were studied (6 patients × 2 groups × 2 exposure moments × 4 experimental conditions).

2.4. Soluble cytokine levels measurement

Levels of soluble IL-2, IFN-γ (Th1 cytokine), IL-4 and IL-5 (Th2 cytokines), IL-10 (Treg cytokine) were analyzed in the cell supernatants by flow cytometry, using the BD Cytometric Bead Array (CBA), human Th1/Th2 Cytokine Kit (Becton Dickinson, BD, San Diego, CA) and the BD CBA Human Soluble Protein Flex Set System (BD) for IL-13 (T_H2 cytokine). Flow cytometric analyses were