



Intranasal immunization with a dominant T-cell epitope peptide of a major allergen of olive pollen prevents mice from sensitization to the whole allergen

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Abstract

Mucosal tolerance induction with vaccines based on peptides representing T-cell epitopes of allergens is a promising way for treating allergic diseases. Ole e 1 is the main allergen of olive pollen, which is an important cause of allergy in Mediterranean countries. The aim of this study was to evaluate the ability of the peptide T109-K130 containing a dominant T-cell epitope of Ole e 1, to modulate the allergen-specific immune response in a prophylactic mouse model. Mice were intranasally treated with the peptide 1 week prior to sensitization with Ole e 1. Blood, lungs and spleens were collected and analysed for immune response. Intranasal pretreatment of mice with the peptide led to suppress serum specific IgE, IgG1 and IgG2a antibody levels, and markedly reduced proliferative T-cell response and Th2-cytokine production, but increased IFN- γ secretion in spleen cell cultures. Increased mRNA IL-10 levels were observed in lungs from pretreated mice. Pathologic alterations of the lung associated with airway inflammation (peribronchial/perivascular infiltrates, eosinophilia and mucus production) were significantly suppressed after pretreatment. Similar results were obtained when mice were sensitized 10 weeks after treatment. Our results demonstrate that intranasal administration of a single T-cell peptide protects mice against subsequent sensitization to the allergen, possibly via IFN- γ and IL-10. This study emphasizes the usefulness of nasal peptide T-based vaccines against allergy.

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1. Introduction

Nowadays specific immunotherapy (SIT) is the only curative treatment for allergy. SIT is performed by injection of increasing doses of the allergen extract to induce immunologic hyporesponsiveness. Despite of the efficacy of SIT, concerns exist with the safety of this form of immunotherapy as well as with the difficulty of standardization of the allergen extract used as vaccine. Consequently, there is strong interest in the

development of novel strategies for allergy. One of the principal approaches is the use of mucosal vaccines which are based on the mucosal tolerance phenomenon (Wiedermann, 2005). Other alternatives are based on the use of allergen derivatives with reduced or no allergenicity as vaccines (Vrtala et al., 2004). These included chemically modified allergens, mutants obtained by protein engineering and synthetic peptides representing immunodominant T-cell epitopes of allergens. Several studies have demonstrated the feasibility of the combination of these two approaches for clinical application into humans in order to reinstate homeostasis toward allergens (review in Larché and Wraith, 2005; Wiedermann, 2005).

Olive tree (*Olea europaea*) pollen is one of the most important causes of pollinosis in Mediterranean countries and some areas of America and Australia, where it is widely cultivated (Liccardi et al., 1996). Ole e 1 has been identified as the most relevant allergen of olive pollen and affects approximately 80%

Abbreviations: Ab, antibody; Ag, antigen; CD, circular dichroism; H&E, hematoxylin/eosin; i.n., intranasal; mAb, monoclonal antibody; OE_{109–130}, Ole e 1_{109–130} peptide; SIT, specific immunotherapy

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of the patients allergic to this pollen (Wheeler et al., 1990). Moreover, it also represents the main sensitizing agent within the protein family of Ole e 1-related allergens (Mothes et al., 2004) and a diagnostic marker for sensitization to Oleaceae pollens (Palomares et al., 2006). Therefore, Ole e 1 has been selected as a model allergen for the development of a novel strategy for the treatment of allergy. Ole e 1 consists of a single polypeptide chain of 145 amino acid residues. This allergen has been purified and sequenced (Villalba et al., 1993), cloned (Villalba et al., 1994) and produced as recombinant protein in the yeast *Pichia pastoris* with biochemical and immunological properties equivalent to those of the natural allergen (Huecas et al., 1999; Quiralte et al., 2000). Previous studies were performed to elucidate the T-cell and B-cell determinants of Ole e 1 by using overlapping synthetic peptides, and reported that the segment T109–K130 showed capability to stimulate T cells from allergic patients (Cárdaba et al., 1998), but displays no IgE-binding capability (Cárdaba et al., 1998; González et al., 2006). Moreover, this region was defined as the most important T-cell epitope of Ole e 1 in humans (Cárdaba et al., 1998). Based on these data, a peptide of 22 amino acids was designed, spanning the amino acids 109–130 of Ole e 1 (OE_{109–130}). This peptide would be a good immunotherapeutic agent for allergy, since it substantially would decrease the risk of IgE-mediated anaphylactic side effects. The aim of this study was to evaluate whether the intranasal (i.n.) administration of OE_{109–130} could efficiently induce tolerance in a prophylactic approach, using a mouse model of allergic sensitization to Ole e 1. For comparison, natural Ole e 1 was also used as vaccine in this study.

2. Materials and methods

2.1. Mice

Female, 6–8-week-old BALB/c mice were obtained from Harlan Interfauna Ibérica SA (Barcelona, Spain). Animals were maintained at the Animal Care Services of the Faculty of Biology (Universidad Complutense, Madrid, Spain), according to the local guidelines for animal care. The studies were approved by the Animal Experimentation Ethics Committee of the Complutense University.

2.2. Peptide and protein

Ole e 1_{109–130} peptide (OE_{109–130}, TVNGTTRTVNPLGF-FKKEALPK) was purchased from Thermo Electron GmbH (Ulm, Germany). Peptide was analysed and purified by reverse-phase HPLC, and checked by mass spectrometry. The purity of the peptide was >90%.

Ole e 1 was purified from olive pollen as described (Villalba et al., 1993).

2.3. Sera, antibodies

Sera from five olive pollen-allergic patients with a positive reaction to Ole e 1 were taken from a population with a clinical history of allergy to olive pollen and a positive skin-prick test and

CAP-FEIA System (Pharmacia AB, Uppsala, Sweden) classes 3–6 to olive pollen. A written informed consent was obtained from all patients. A pool of sera from non-allergic individuals was used as negative control. The sera from allergic and non-allergic individuals were kindly donated by Drs. M. Martín-Esteban and C. Pascual (Hospital La Paz, Madrid, Spain).

F2 y F7 monoclonal antibodies (mAbs) against Ole e 1 were provided by Dr. D. Barber (ALK-Abelló, Madrid, Spain) and R. van Ree (CLB, Amsterdam, The Netherlands), respectively. Polyclonal serum against Ole e 1 was prepared in rabbit as reported (Huecas et al., 1999).

2.4. Dot-blot

Protein and peptide were directly applied onto nitrocellulose membranes (Hybond ECL, Amersham, Buckinghamshire, UK). Membranes were incubated with Ole e 1-specific Abs or sera from olive-pollen allergic patients as described (González et al., 2002).

2.5. Circular dichroism (CD) analyses

CD spectra were obtained on a Jasco J-715 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) fitted with a 150-W xenon lamp and connected to a Weslab RTE-111 thermostabilizer bath. Far-UV spectra were registered in the range of 190–250 nm using optical-path cell of 0.1 cm. Samples were analysed in 20 mM sodium phosphate, pH 7.2, at 25 °C. Mean residue mass ellipticities were calculated based on 110 as the average molecular mass/residue, obtained from the amino acid composition, and expressed in terms of θ (deg cm² dmol^{−1}). Final spectra were corrected by subtracting the corresponding baseline spectrum obtained for the buffer under identical conditions. Secondary structure estimations were performed by computer fit according to the method of Perczel et al. (1991).

2.6. Sensitization and airway challenge

Mice ($n=5$ /group) were sensitized by intraperitoneal injections with 1 μ g Ole e 1 adsorbed to 2 mg Al(OH)₃ (Sigma Chemical Co., St. Louis, MO) in 150 μ l of PBS on days 0 and 7. On day 14, mice were challenged by i.n. administration of 10 μ g of Ole e 1 in PBS on 3 consecutive days. Twenty-four hours after the last i.n. challenge, lung histology was examined. Blood samples were collected from the retro-orbital plexus (terminal bleeding) 7 days after the last immunization. Individual sera were prepared and stored at −20 °C until analysed.

2.7. Prophylactic protocol

Mice ($n=5$ /group) were i.n. pretreated three times on consecutive days with Ole e 1 or OE_{109–130} (Fig. 2A). In a pilot experiment, several i.n. pretreatments were performed to find the optimal dose of antigen administration: 50, 5, 0.5 μ g. Immunomodulation was achieved by i.n. application of 5 μ g Ole e 1 or 50 μ g OE_{109–130}, and were, therefore, chosen as the optimal doses in the present study. One week later, mice were

sensitized to Ole e 1 as described above (short-term protocol). Sampling and analyses were performed as mentioned above. The control mice were sham-pretreated with PBS only, and naïve mice were given no treatment. For long-term experiments, sensitization to Ole e 1 was started 10 weeks after pretreatment (Fig. 5A).

2.8. Histopathology

Twenty-four hours after the last allergen exposure, lungs were collected and fixed in 10% paraformaldehyde in PBS. After embedding in paraffin, the tissues were cut into 5 µm sections and stained with hematoxylin/eosin (H&E). Lung sections were observed under light microscopy (Axiophot 2 model, Zeiss, Jena, Germany) and photographed with a digital camera (Spot Insight Color, Diagnostic Instruments, MI, USA). For each mouse, 10 airway sections were assessed for severity of the cellular inflammatory response and mucus occlusion by morphometric analysis, by individuals blinded to the protocol design.

2.9. Determination of allergen-specific IgG1 and IgG2a, and total IgE in serum of mice

Antigen (Ag)-specific Abs were measured by ELISA as described (Batanero et al., 2002). Serum samples were diluted 1:25,000 for IgG1 and 1:400 for IgG2a. Bound mouse immunoglobulins were detected using alternatively peroxidase-conjugated rabbit anti-mouse IgG1 (diluted 1:5000), or anti-mouse IgG2a (diluted 1:5000) Abs (Rockland, Gilbertsville, PA, USA) and *o*-phenylenediamine chloride (Merck & Co. Inc., Whitehouse Station, NJ, USA) as substrate (González et al., 2002). Optical density was measured at 492 nm.

Total IgE (diluted 1:40) Abs were measured by sandwich ELISA using the OptEIA mouse IgE set (PharMingen, San Diego, CA, USA) according to the manufacturer's instructions. In our mouse model, it has been showed that specific IgE can be estimated by measuring total IgE levels in serum (Batanero et al., 2002).

2.10. Proliferation and cytokine assays

Seven days after last immunization, spleens were removed aseptically, minced, filtered through sterile filters, and a cell suspension was prepared as described (Batanero et al., 2002).

For T-cell proliferation assays, spleen cells (2×10^6 cells/ml, 0.2 ml/well) were stimulated or not with concanavalin A (2–0.02 µg/ml; Sigma), Ole e 1 (2–0.02 µg/ml) or the peptide (2–0.02 µg/ml) for 96 h in a 96-round bottom plates (Costar, Cambridge, MA, USA). The cultures were pulsed with 1 µCi/well [^{3} H]-thymidine (Amersham) for 16 h and cells were harvested on microbeta filter mats using a 96-well harvester, and proliferative response was measured by beta plate scintillation counting. The stimulation index was calculated as the ratio of cpm of the unstimulated control and allergen-stimulated cells.

To measure the cytokine production, spleen cells were cultured in 48-well plates (Costar) at 10^7 cells/ml (0.5 ml/well) and stimulated or not with concanavalin A (2 µg/ml) or Ole

e 1 (2 µg/ml). Supernatants were taken after 48 or 72 h and stored at -20°C until analyses. Levels of IL-4, IL-5, IL-10 and IFN- γ were measured by ELISA kits (Pharmingen). Values in pg/ml were calculated according to standard curves obtained with recombinant mouse cytokines. The sensitivity of the assays was 5 pg/ml for IL-4, 60 pg/ml for IL-5, 15 pg/ml for IL-10 and 25 pg/ml for IFN- γ .

2.11. RNA isolation and RT-PCR assays

Lungs and spleens were homogenized and total RNA was isolated using RNAqueous-4PCR kit (Ambion Europe, Ltd., Cambridgeshire, UK) according to the manufacturer's instructions. Afterwards, the samples were treated with DNase and reverse transcribed into cDNA using Retroscript kit (Ambion). Primers specific for IL-10 (forward 5'- CCCAGAAATCAAGGAGC-ATTTG- 3'; reverse 5'-CATGTATGCTTCTATGCAGTTG-3'), TGF- β (forward 5'- ACCGCAACAACGCCATCTAT- 3'; reverse 5'- GTAACGCCAGGAATTGTTGC- 3') and β -actin (forward 5'- GTGGGCCCGCCCTAGGCACCAGG - 3'; reverse 5'- CTCTTTGATGTCACGCACGATTC-3') were designed and synthesized by Sigma. PCR amplification was conducted for 35 cycles at 95°C for 60 s, 54°C for 30 s, and 72°C for 60 s, followed by one cycle at 72°C for 10 min. PCR products were separated by electrophoresis in 1.5% agarose gels and visualized with ethidium bromide and UV illumination. The density of the bands on the gel was measured using an image analyser system (UVI Tech, Cambridge, UK). After normalization of the data according to the expression of β -actin mRNA, relative levels of cytokine mRNA were calculated.

3. Results

3.1. OE_{109–130} is an important T-cell epitope of Ole e 1 in BALB/c mice

Before examining the immunosuppressive ability of OE_{109–130}, the peptide was checked for T-cell reactivity using spleen cells obtained from mice sensitized with Ole e 1 (Fig. 1A). Lymphocytes proliferated to either Ole e 1 or OE_{109–130}. The proliferative response of the peptide was estimated to be 50% of the whole protein. In view of this result, OE_{109–130} peptide clearly appeared to be an important T-cell epitope of Ole e 1 in mice as it has been reported in humans (Cárdaba et al., 1998).

The presence of an IgG-epitope and the lack IgE-binding capacity reported for the segment T109-K130 of Ole e 1 was confirmed for OE_{109–130} (Fig. 1B). The peptide was recognized by the F2 mAb and a polyclonal antiserum both specific to Ole e 1, but not by IgE from patients allergic to this allergen. OE_{109–130} was not recognized by the F7 mAb, assuring the specificity of the assay. The peptide adopted mostly random conformation in aqueous solution as demonstrated by CD analysis (Fig. 1C). These results confirmed the feasibility of this peptide to be used in a prophylactic approach for tolerance induction in a mouse model of allergy.

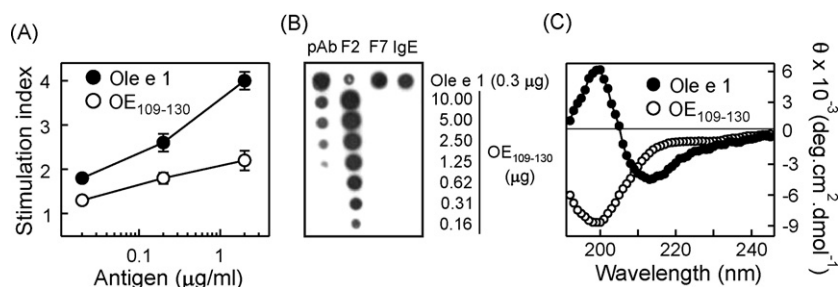


Fig. 1. Immunological characterization of OE₁₀₉₋₁₃₀. (A) T-cell reactivity in spleen cell cultures from Ole e 1-sensitized mice after *in vitro* stimulation with Ole e 1 or OE₁₀₉₋₁₃₀. Proliferative responses were measured by [³H]thymidine-incorporation and indicated as stimulation index. (B) Dot blot of the Ig-binding capacity of the peptide OE₁₀₉₋₁₃₀ in comparison with Ole e 1 using one polyclonal antiserum (pAb) and two mAbs (F2 and F7) against Ole e 1, and serum IgE from allergic patients (IgE). (C) CD spectra of OE₁₀₉₋₁₃₀ and Ole e 1 in the far-UV region.

3.2. Intranasal pretreatment with OE₁₀₉₋₁₃₀ suppresses Ole e 1-specific IgE response as efficiently as the whole protein

Intranasal pretreatment with the whole protein was highly effective in suppressing Ole e 1-specific IgE production (Fig. 2B). IgE Ab levels were up to four times lower in Ole e 1-pretreated mice compared to the sham-pretreated animals. Allergen-specific IgG1 and IgG2a Ab levels were increased by 54% and 44%, respectively, after *i.n.* pretreatment (Fig. 2B).

Intranasal administration of OE₁₀₉₋₁₃₀ before sensitization to Ole e 1 reduced the expected specific-IgE response as the whole protein (Fig. 2B). This inhibition was 45% when compared to sham-pretreated controls. In contrast to the Ole e 1-pretreatment, the down-regulation of specific-IgE was accompanied by a parallel decrease by 40% and 79% of the specific-IgG1 and IgG2a responses, respectively, when compared to the sham-pretreated controls (Fig. 2B). From these results, it can be inferred that *i.n.* pretreatment with OE₁₀₉₋₁₃₀ induces tolerance in IgE and IgG responses, as inhibited both Th1 and Th2 Abs specific to Ole e 1.

3.3. Intranasal pretreatment with OE₁₀₉₋₁₃₀ inhibits the proliferative response to Ole e 1 and induces IFN- γ production

The lymphoproliferative response to Ole e 1 was measured in pooled spleen cell cultures. Spleen cell of Ole e

1-pretreated mice proliferated markedly less upon stimulation with the allergen compared with that of sham-pretreated group (Fig. 3A). Intranasal pretreatment with the peptide also resulted in a markedly reduction of the lymphoproliferative response (Fig. 3A). Thus, *i.n.* administration of OE₁₀₉₋₁₃₀ suppressed T-cell proliferative response against Ole e 1 in a similar extent than the whole protein.

To characterize the *in vitro* cytokine profile, IL-4, IL-5, IL-10 and IFN- γ levels were measured in pooled spleen cell cultures. IL-4, IL-5, IL-10 and IFN- γ production was significantly reduced in spleen cell cultures of Ole e 1-pretreated mice in comparison to the sham-pretreated controls (Fig. 3B). Pretreatment with the peptide resulted in a significant inhibition of IL-4, IL-5 and IL-10 production whereas IFN- γ levels were markedly increased (Fig. 3B). These results indicate that *i.n.* peptide-pretreatment induces a shift from Th2 to Th1-cytokine profile.

3.4. Intranasal pretreatment with OE₁₀₉₋₁₃₀ leads to increase the expression of IL-10 mRNA in lung, but no TGF- β mRNA

The expression of IL-10 mRNA was increased 2.6-fold in lung tissues of mice that had been *i.n.* pretreated with OE₁₀₉₋₁₃₀ compared to sham-pretreated controls (Fig. 3C). In contrast, no significant changes in IL-10 mRNA expression were detected

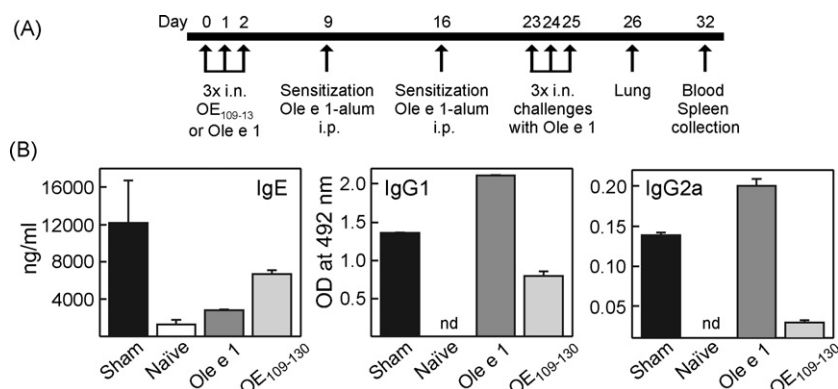


Fig. 2. Allergen-specific Ab responses in sera of OE₁₀₉₋₁₃₀-pretreated mice compared to Ole e 1-pretreated group. (A) Experimental protocol used. BALB/c mice were treated *i.n.* for three consecutive days either with OE₁₀₉₋₁₃₀ or with Ole e 1 one week prior to sensitization with the allergen. Sham-control mice received only PBS. (B) Ole e 1-specific IgE, IgG1, and IgG2a Ab levels in sera were determined by means of ELISA. Data are presented as mean $\pm$ S.D. values of individual mice (n = 5). Experiments were repeated twice with similar results. nd, not detected.

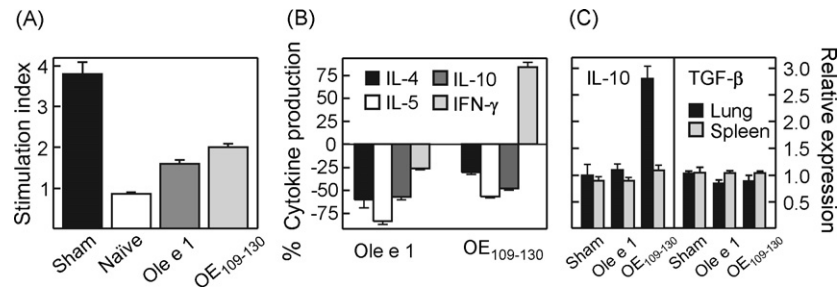


Fig. 3. T-cell reactivity in spleen cell cultures from pretreated mice. (A) Proliferative responses to Ole e 1 ($2 \mu\text{g/ml}$) were measured by [^3H]thymidine-incorporation and indicated as stimulation index. (B) Cytokine production in spleen cell cultures was determined after *in vitro* stimulation with Ole e 1 by ELISA. Data are expressed as percentage of increase or decrease compared with sham-pretreated values. (C) IL-10- and TGF- β mRNA expression in lung and spleen cells from Ole e 1- and peptide-pretreated mice compared to sham-pretreated controls. Data are expressed as cytokine/ β -actin ratio. Experiments were repeated twice with similar results.

after pretreatment with Ole e 1. TGF- β mRNA was also analysed, and no significant changes in its expression were detected in both OE₁₀₉₋₁₃₀ and Ole e 1-pretreated mice compared to sham-pretreated group (Fig. 3C). The expression of IL-10 and TGF- β mRNA in spleen remained unchanged in either of the pretreated groups (Fig. 3C).

3.5. Intranasal pretreatment with OE₁₀₉₋₁₃₀ suppressed airway inflammation

Twenty-four hours following the last i.n. Ole e 1 challenge, lung tissues were obtained from each experimental group to assess the effect of OE₁₀₉₋₁₃₀ pretreatment on airway inflammation. Histological examination revealed that Ole e 1-sensitization of mice promoted epithelial cell damage with goblet hyperplasia and cellular infiltration around blood vessels and airways (Fig. 4C) in comparison to naïve mice (Fig. 4A). Mucus hypersecretion and occlusion of the airways were prominent histopathologic feature of the sham-pretreated mice (Fig. 4D). Eosinophils and lymphocytes were the predominant infiltrating cells (Fig. 4F). Intranasal pretreatment with the peptide significantly reduced cellular infiltration in the lung of Ole e 1-

sensitized/challenged mice (Fig. 4G), but to a lesser extent than for i.n. Ole e 1-pretreatment (Fig. 4E). The cellular infiltrate was predominantly characterized by mononuclear cells but not eosinophils (Fig. 4H). Mucus release was completely inhibited by i.n. pretreatment with either the peptide or Ole e 1 (Fig. 4H). These data indicate that i.n. administration of OE₁₀₉₋₁₃₀ can attenuate lung inflammation induced by the allergen challenge as Ole e 1 does.

3.6. Peptide pretreatment induces a long-term immunosuppressive effect

After the long-term protocol, both OE₁₀₉₋₁₃₀ and Ole e 1 pretreatments suppressed the serum specific-IgE Ab levels to the same extent as after the short-term protocol compared to sham-pretreated mice (Fig. 5B). In contrast with the short-term approach, specific-IgG1 Ab levels did not significant changes after a long-term interval (Fig. 5B). Pretreatment with the peptide strongly decreased the specific-IgG2a Ab levels after both the short-term and the long-term protocols.

After a long interval between treatment and sensitization, lymphoproliferative response and cytokine production (IL-4, IL-

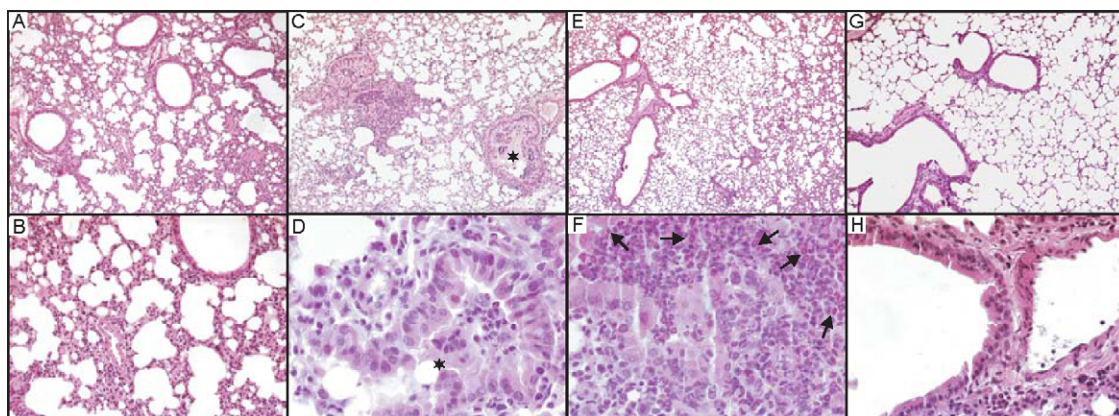


Fig. 4. Representative photomicrographs of lung sections from each group. Naïve mice (A and B): (A) normal airway mucosa and surrounding parenchyma; (B) airway mucosa showing low cuboidal cells and absence of peribronchiolar inflammatory infiltrates. Sham mice (C, D and F): (C) severe peribronchial and perivascular infiltration of inflammatory cells and large accumulation of mucus in the airway lumen is observed (*); (D) marked thickening of airway epithelium and goblet cell hyperplasia, and (F) intensive mixed cellular influx of eosinophils ($\rightarrow$) and other inflammatory cells. Mice pretreated with Ole e 1 (E) or OE₁₀₉₋₁₃₀ (G): (E and G) minimal cellular infiltration around the blood vessels and airways are found; (H) eosinophils in the lung interstitial tissue and mucus secretion in the airways are not observed. Lung sections were stained with H&E. Magnification: (A and G) $\times 10$; (C and E) $\times 5$; (B) $\times 20$; (D, F and H) $\times 63$.

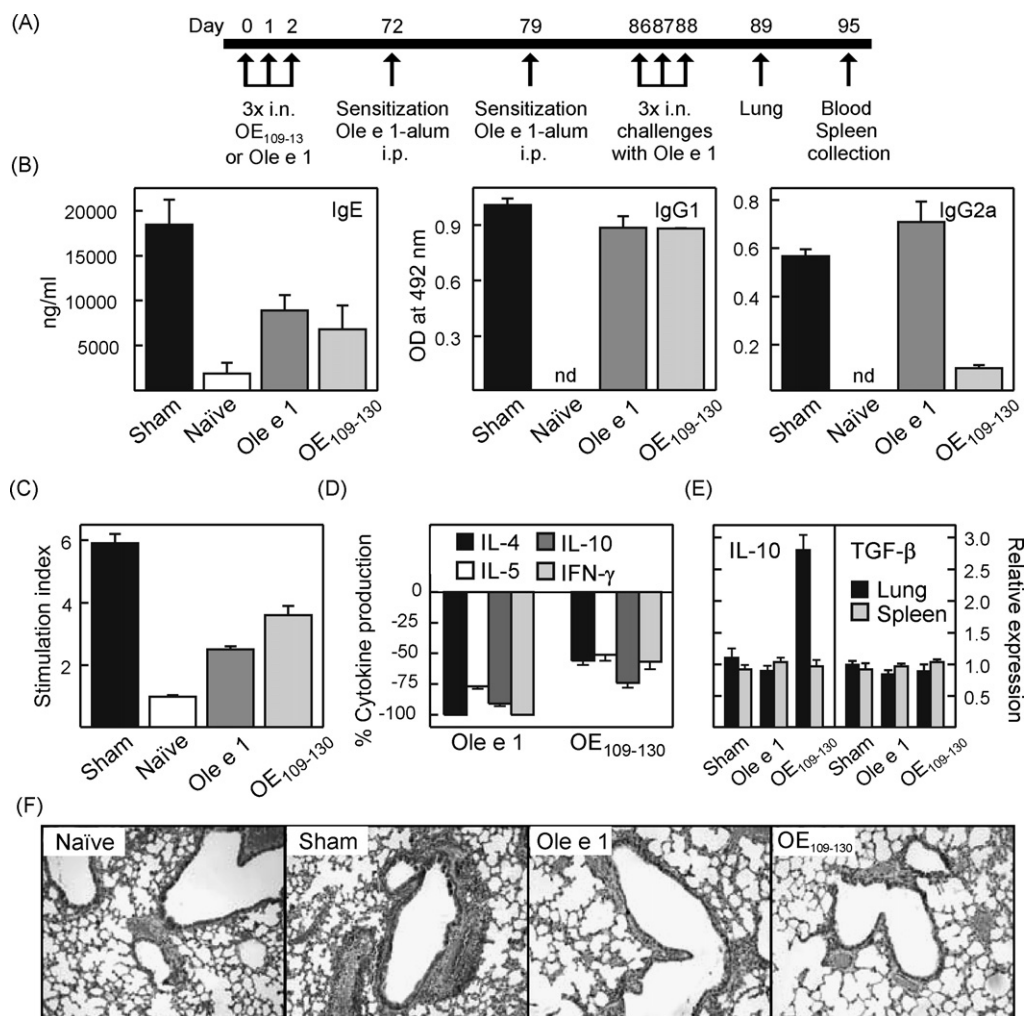


Fig. 5. Long-term immunosuppression effects after i.n. pretreatment with OE₁₀₉₋₁₃₀ or with Ole e 1. (A) Experimental protocol used. Mice ($n = 5$) were i.n. pretreated with OE₁₀₉₋₁₃₀, Ole e 1 or sham-pretreated and then sensitized to Ole e 1 10 weeks later. (B) Ole e 1-specific Ab levels were measured by ELISA. Values are expressed as mean $\pm$ S.D. from five mice per group. Proliferative response (C) and cytokine production (D) in spleen cell cultures stimulated *in vitro* with Ole e 1 ($2 \mu\text{g/ml}$). Results of proliferation assays are expressed as stimulation index. Cytokine levels were measured by ELISA. Data are expressed as percentage of increase or decrease compared with sham-pretreated values. (E) IL-10- and TGF- β mRNA expression in lung and spleen cells from Ole e 1- and peptide-pretreated mice compared to sham-pretreated controls. Data are expressed as cytokine/ β -actin ratio. (F) Representative photomicrographs of lung sections from each group. Lung sections were stained with H&E. Magnification, $\times 10$. Experiments were repeated twice with similar results.

5 and IL-10 and IFN- γ) were reduced in spleen cell cultures of pretreated mice compared to the sham-pretreated controls (Fig. 5C and D).

The expression of IL-10 mRNA in lung tissues increased in the OE₁₀₉₋₁₃₀-pretreated mice, but remained unchanged in the animals pretreated with Ole e 1 compared to those of sham-pretreated controls in the long-term protocol as observed for short-term experiments (Fig. 5E). The expression of IL-10 mRNA in spleen cells did not, however, differ among the groups. No significant differences of TGF- β mRNA levels were detected in lung and spleen cells of pretreated mice compared to the sham-pretreated group in both the long-term and the short-term protocols.

Finally, histological analysis revealed that after a long-term interval, i.n. pretreatment with the peptide as well as with Ole e 1 significantly reduced airway inflammation that occurs in the sham-pretreated mice (Fig. 5F), according to the blocking of the

peribronchial/perivascular infiltration, influx of eosinophils into the lungs and mucus release, as it was shown for the short-term protocol. Thus, i.n. OE₁₀₉₋₁₃₀ pretreatment induces a long-term immunosuppressive effect as efficiently as Ole e 1.

4. Discussion

In this study, we analysed the ability of an i.n. OE₁₀₉₋₁₃₀ peptide-based vaccine to modulate the immune response and to prevent the synthesis of IgE Abs and airway inflammation in a mouse model of Ole e 1-sensitization. We identified the peptide OE₁₀₉₋₁₃₀ as an important T-cell epitope in BALB/c mice that was also described as the most important T-cell epitope of Ole e 1 in humans (Cárdaba et al., 1998). It was demonstrated that prophylactic i.n. administration of the peptide suppressed allergen-specific-IgE Ab levels, T-cell proliferation and Th2-cytokine production *in vitro*, and airway inflamma-

tion as efficiently as the whole protein. The major advantage of this peptide compared to Ole e 1 is its lack of allergenicity, providing a safe tolerogen for the treatment of allergy. Three i.n. administrations of OE_{109–130} performed on consecutive days were enough for inhibiting the outcome of specific-IgE response in mice after sensitization with Ole e 1 allergen, indicating that a protocol of prolonged exposition to the peptide is not necessary to inhibit the development of allergic immune response *in vivo*. Moreover, peptide efficiently inhibited both specific-IgG1 and IgG2a Ab productions whereas i.n. pretreatment with Ole e 1 leads to a strong production of specific-IgG2a and IgG1 Abs. The inhibition of IgE synthesis simultaneous with the increase on IgG1 production in Ole e 1-pretreated mice pointed out differences in the control of Ag-specific IgE and IgG1 class switch by T-cell in agreement with the findings of other authors (van Halteren et al., 1997). A similar dissociation between IgE and IgG4 (the equivalent of mouse IgG1) occurs in humans after allergen immunotherapy (Frew, 2003).

In this model of i.n. tolerance in naïve mice induced by the peptide, the inhibition of T-cell proliferation was associated with the reduction of Th2-type cytokine secretion and the increase of that of Th1-type. By contrast, Ole e 1 suppressed production of both Th1- and Th2-cytokines in the prophylactic model. The differences described for the immune responses induced by OE_{109–130} and Ole e 1 may be related to the nature of the Ag *per se* as has been indicated by others authors (Wiedermann et al., 1999a; Hufnagl et al., 2002).

Lung tissue inflammation was noticeably reduced in both Ole e 1- and OE_{109–130}-pretreated mice compared to sham-pretreated mice, suggesting that both pretreatments influenced not only the systemic immune response but also the local immune response. These results support the idea that OE_{109–130} may be useful in the treatment of allergic patients by attenuation of the main allergic symptoms.

The expression of IL-10 mRNA was up-regulated in lung only after i.n. pretreatment with OE_{109–130}, and this was observed together with the reduction of the airway inflammation. This indicates that the production of IL-10 could be associated with the peptide-induced tolerance observed in this model, as it has been reported in several murine models of allergy (review in Akdis et al., 1998; Wiedermann, 2005). Furthermore, the elevation of IL-10 following immunotherapy has been reported in human clinical trials (Akdis et al., 1998; Fellrath et al., 2003; Jutel et al., 2003; Nouri-Aria et al., 2004; Oldfield et al., 2002). However, there is not evidence from our results that TGF- β , a key cytokine in several models of tolerance induction (Wiedermann et al., 1999b; Winkler et al., 2003), might be involved in the immunomodulation observed in our model, since no differences in TGF- β mRNA levels were detected among groups. The tolerance mechanism induced by the peptide pretreatment seemed to differ from that induced by the whole Ole e 1. Only the peptide led to increase IL-10 mRNA, along with an increase in IFN- γ protein levels, indicating that both IL-10 and IFN- γ may operate in concert to tolerance induced by i.n. peptide pretreatment. Subsequent to this, evidences from several studies indicate that successful allergen immunotherapy is associated with an increase either IL-10 alone or both IL-10 and IFN- γ (Akdis et

al., 1998; Fellrath et al., 2003; Oldfield et al., 2002; Till et al., 2004).

Finally, the i.n. pretreatment with both molecules induces specific long-lasting protection against the development of allergic immune response *in vivo*. Similar results have been observed in human studies (Creticos et al., 2006; Durham et al., 1999).

5. Conclusions

This study showed that prophylactic i.n. administration of an immunodominant T-cell epitope peptide derived of Ole e 1 may substitute for the whole protein in order to suppress three important immunological parameters occurring after subsequent sensitization and respiratory challenge with the allergen: specific-IgE Abs, Th2-cytokines and airway inflammation. Despite the clinical relevance to test, the effects of the i.n. treatment with the peptide in already primed mice, the possibility of using prophylactic allergy vaccines for early prevention in atopic individuals or children at risk has been proposed (Holt, 1994; Valenta, 2002; Wiedermann, 2005). These results suggest that OE_{109–130} shows several features which make it an attractive candidate for the development of safe and effective prophylactic and therapeutic strategies against olive pollen allergy.

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References

- Akdis, C.A., Blesken, T., Akdis, M., Wuthrich, B., Blaser, K., 1998. Role of interleukin 10 in specific immunotherapy. *J. Clin. Invest.* 102, 98–106.
- Batanero, E., Barral, P., Villalba, M., Rodríguez, R., 2002. Sensitization of mice with olive pollen allergen Ole e 1 induces a Th2 response. *Int. Arch. Allergy Immunol.* 127, 269–275.
- Cárdaba, B., del Pozo, V., Jurado, A., Gallardo, S., Cortegano, I., Arrieta, I., del Amo, A., Tramón, P., Florido, F., Sastre, J., Palomino, P., Lahoz, C., 1998. Olive pollen allergy: searching for immunodominant T-cell epitopes on the Ole e 1 molecule. *Clin. Exp. Allergy* 28, 413–422.
- Creticos, P.S., Schroeder, J.T., Hamilton, R.G., Balcer-Whaley, S.L., Khattig-navong, A.P., Lindblad, R., Li, H., Coffman, R., Seyfert, V., Eiden, J.J., Broide, D., Immune tolerance network group, 2006. Immunotherapy with a ragweed-toll-like receptor 9 agonist vaccine for allergic rhinitis. *N. Engl. J. Med.* 355, 1445–1455.
- Durham, S.R., Walker, S.M., Varga, E.M., Jacobson, M.R., O'Brien, F., Noble, W., Till, S.J., Hamid, Q.A., Nouri-Aria, K.T., 1999. Long-term clinical efficacy of grass-pollen immunotherapy. *N. Engl. J. Med.* 341, 468–475.
- Fellrath, J.-M., Kettner, A., Dufour, N., Frigerio, C., Schneeberger, D., Leimgruber, A., Corradin, G., Spertini, F., 2003. Allergen-specific T-cell tolerance induction with allergen-derived long synthetic peptides: results of a phase I trial. *J. Allergy Clin. Immunol.* 111, 854–861.
- Frew, A.J., 2003. Immunotherapy of allergic disease. *J. Allergy Clin. Immunol.* 111, S712–S719.
- González, E., Villalba, M., Lombardero, M., Aalbers, M., van Ree, R., Rodríguez, R., 2002. Influence of the 3D-conformation, glycan component and microheterogeneity on the epitope structure of Ole e 1, the major olive allergen. Use of recombinant isoforms and specific monoclonal antibodies as immunological tools. *Mol. Immunol.* 39, 93–101.

- González, E., Villalba, M., Quirarte, J., Batanero, E., Roncal, F., Albar, J.P., Rodríguez, R., 2006. Analysis of IgE and IgG B-cell immunodominant regions of Ole e 1, the main allergen from olive pollen. *Mol. Immunol.* 43, 570–578.
- Holt, P.G., 1994. A potential vaccine strategy for asthma and allied atopic diseases during early childhood. *Lancet* 344, 456–458.
- Huecas, S., Villalba, M., González, E., Martínez-Ruiz, A., Rodríguez, R., 1999. Production and detailed characterization of biologically active olive pollen allergen Ole e 1 secreted by the yeast *Pichia pastoris*. *Eur. J. Biochem.* 261, 539–545.
- Hufnagl, K., Winkler, B., Baier, K., Valenta, R., Kraft, D., Wiedermann, U., 2002. Induction of mucosal tolerance by co-application of recombinant Bet v 1, the major birch pollen allergen, recombinant Phl p 1 and Phl p 5, major grass pollen allergens, in poly-sensitized mice. *Allergy* 57 (Suppl. 73), 49 (Abstract).
- Jutel, M., Akdis, M., Budak, F., Aebischer-Casaulta, C., Wrzyszczy, M., Blaser, K., Akdis, C.A., 2003. IL-10 and TGF- β cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur. J. Immunol.* 33, 1205–1214.
- Larché, M., Wraith, D.C., 2005. Peptide-based therapeutic vaccines for allergic and autoimmune diseases. *Nat. Med.* 11, S69–S76.
- Liccardi, G., D'Amato, M., D'Amato, G., 1996. *Oleaceae* pollinosis: a review. *Int. Arch. Allergy Immunol.* 111, 210–217.
- Mothes, N., Horak, F., Valenta, R., 2004. Transition from a botanical to a molecular classification in tree pollen allergy: implications for diagnosis and therapy. *Int. Arch. Allergy Immunol.* 135, 357–373.
- Nouri-Aria, K.T., Wachholz, P.A., Francis, J.N., Jacobson, M.R., Walker, S.M., Wilcock, L.K., Staple, S.O., Aalberse, R.C., Till, S.J., Durham, S.R., 2004. Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. *J. Immunol.* 172, 3252–3259.
- Oldfield, W.L., Larché, M., Kay, A.B., 2002. Effect of T-cell peptides derived from Fel d 1 on allergic reactions and cytokine production in patients sensitive to cats: a randomised controlled trial. *Lancet* 360, 47–53.
- Palomares, O., Swoboda, I., Villalba, M., Balic, N., Spitzauer, S., Rodríguez, R., Valenta, R., 2006. The major allergen of olive pollen Ole e 1 is a diagnostic marker for sensitization to *Oleaceae*. *Int. Arch. Allergy Immunol.* 141, 110–118.
- Perczel, A., Hollosi, M., Tusnady, G., Fasman, G.D., 1991. Convex constraint analysis: a natural deconvolution of circular dichroism curves of proteins. *Protein Eng.* 4, 669–679.
- Quirarte, J., González, E., Arias de Saavedra, J.M., Villalba, M., Florido, J.F., Saénz de San Pedro, B., Rodríguez, R., 2000. Immunological activity of recombinant Ole e 1 in patients with *Olea europaea* pollinosis. *Int. Arch. Allergy Immunol.* 122, 101–107.
- Till, S.J., Francis, J.N., Nouri-Aria, K., Durham, S.R., 2004. Mechanism of immunotherapy. *J. Allergy Clin. Immunol.* 113, 1025–1034.
- van Halteren, A.G., van der Cammen, M.J., Cooper, D., Savelkoul, H.F., Kraal, G., Holt, P.G., 1997. Regulation of antigen-specific IgE, IgG1, and mast cell responses to ingested allergen by mucosal tolerance induction. *J. Immunol.* 159, 3009–3015.
- Valenta, R., 2002. The future of antigen-specific immunotherapy of allergy. *Nat. Rev. Immunol.* 2, 446–453.
- Villalba, M., Batanero, E., López-Ortín, C., Sánchez, M.L., Monsalve, R.I., González de la Peña, M.A., Lahoz, C., Rodríguez, R., 1993. The amino acid sequence of Ole e I, the major allergen from olive tree (*Olea europaea*) pollen. *Eur. J. Biochem.* 216, 863–869.
- Villalba, M., Batanero, E., Monsalve, R.I., González de la Peña, M.A., Lahoz, C., Rodríguez, R., 1994. Cloning and expression of Ole e I, the major allergen from olive tree pollen: polymorphism analysis and tissue specificity. *J. Biol. Chem.* 269, 15217–15222.
- Vrtala, S., Focke-Tejkl, M., Swoboda, I., Kraft, D., Valenta, R., 2004. Strategies for converting allergens into hypoallergenic vaccine candidates. *Methods* 32, 313–320.
- Wheeler, A.W., Hickman, B.E., Fox, B., 1990. Heterogeneity of a major allergen from olive (*Olea europaea*) pollen. *Mol. Immunol.* 27, 631–636.
- Wiedermann, U., 2005. Prophylaxis and therapy of allergy by mucosal tolerance induction with recombinant allergens or allergen constructs. *Curr. Drug Targets Inflamm. Allergy* 4, 577–583.
- Wiedermann, U., Jahn-Schmid, B., Bohle, B., Repa, A., Renz, H., Kraft, D., Ebner, C., 1999b. Suppression of antigen-specific T- and B-cell responses by intranasal or oral administration of recombinant Bet v 1, the major birch pollen allergen, in a murine model of type I allergy. *J. Allergy Clin. Immunol.* 103, 1202–1210.
- Wiedermann, U., Jahn-Schmid, B., Lindblad, M., Rask, C., Holmgren, J., Kraft, D., Ebner, C., 1999a. Suppressive versus stimulatory effects of allergen/cholera toxoid (CTB) conjugates depending on the nature of the allergen in a murine model of type I allergy. *Int. Immunol.* 11, 1717–1724.
- Winkler, B., Bolwig, C., Seppälä, U., Spangfort, M.D., Ebner, C., Wiedermann, U., 2003. Allergen-specific immunosuppression by mucosal treatment with recombinant Ves v 5, a major allergen of *Vespula vulgaris* venom, in a murine model of wasp venom allergy. *Immunology* 110, 376–385.