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The alpha and beta subchain of Amb a 1, the major ragweed-pollen allergen show divergent reactivity at the IgE and T-cell level

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ABSTRACT

Ragweed is one of the most important pollen allergens in North America and parts of Europe. Although the major allergen Amb a 1 was isolated and cloned in 1991, recombinant Amb a 1 was not explored further to improve diagnosis and specific immunotherapy of ragweed-pollen allergy. In the present study the immunological properties of natural Amb a 1 and its proteolytical cleavage products was investigated in detail and compared with recombinant produced Amb a 1 variants.

Characterization of natural Amb a 1 and the identification of its proteolytic fragments, designated Amb a 1α and Amb a 1β, was performed by N-terminal sequencing and mass spectroscopy. Amb a 1 and fragments were further produced in *Escherichia coli*, purified, and immunologically characterized. Amb a 1-specific T-cell cultures were used to compare the T-cell response to the different Amb a 1 variants.

Divergent immunological properties of Amb a 1α (aa 181–396) and Amb a 1β (aa 26–180) were revealed. Amb a 1β contained important IgE epitopes, whereas Amb a 1α showed low IgE binding. When compared to natural Amb a 1, all recombinant variants possessed >100-fold reduced IgE-mediated mediator release activity. At the T-cell level recombinant and natural Amb a 1 stimulated comparable T-cell responses and the T-cell reactivity was largely directed to the C-terminal part. The results demonstrated that recombinant Amb a 1α behaves as hypoallergen with reduced IgE binding but preservation of the major T-cell reactivity. In addition, recombinant Amb a 1α can be easily purified to homogeneity in large quantity and therefore represents an ideal candidate for specific immunotherapy.

1. Introduction

Pollen of short ragweed (*Ambrosia artemisiifolia*) is the single most seasonal allergen in North America and parts of Europe, affecting up to 36 million individuals. Among several allergens described in ragweed pollen, Amb a 1 has been identified as its major allergen (Wopfner et al., 2005). Amb a 1 is an acidic, single-chain 397 residue protein with a molecular weight of approximately 38 kDa (King et al., 1981) and belongs the pectate lyase family (Rafnar et al., 1991). The natural protein undergoes proteolysis during purification resulting in two chains, designated α- and β-chain (King et al., 1974). The 26 kDa α-chain has been reported to associate non-covalently with the 12 kDa β-chain (King et al., 1974, 1981). It has been demonstrated that chemical modifications of the Amb a 1, including reduction and alkylation of disulfide bonds, urea denaturation and renaturation, or succinylation of lysine residues, reduce its IgE reactivity (King, 1976; Smith et al., 1988). The cDNA coding for Amb a 1 was isolated from ragweed pollen in 1991 by Rafnar et al. (1991). Four isoforms of Amb a 1 have been described and sequence comparisons revealed about 80% sequence identity (Griffith et al., 1991; Rafnar et al., 1991). Despite the importance of Amb a 1, reports on the immunological characteristics of the recombinant protein have been extremely rare (Bond et al., 1991; Griffith et al., 1991;

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Rafnar et al., 1991; Wopfner et al., 2008a). Detailed studies of T and B cell epitopes of Amb a 1 as well as a complete characterization of the molecule are required for the rational development of improved reagents for diagnosis and immunotherapy of ragweed-pollen allergy. Current therapeutic options are mainly limited to symptomatic therapies and conventional allergen-specific immunotherapy (SIT), which is performed with crude allergen extracts consisting of a mixture of allergenic and non-allergenic components, difficult to standardize and bearing a risk of IgE-mediated side effects (Ferreira et al., 2006; Holm et al., 2004; Larche et al., 2006; Wallner et al., 2007). Nowadays, a large panel of recombinant allergens has become available which allow the development of hypoallergens, molecules with reduced allergenic activity and retained immunogenicity. Initial immunotherapy trials using hypoallergenic molecules have shown great potential to improve immunotherapy in the near future (Creticos et al., 2006; Ferreira et al., 2006; Gafvelin et al., 2007; Kahlert et al., 2008; Linhart et al., 2008; Niederberger et al., 2004). Various allergens have been used in the development of novel vaccines for the treatment of pollen allergy while the major ragweed-pollen allergen remained poorly characterized (Jutel et al., 2005; Niederberger et al., 2004).

In the present study the immunological properties of natural Amb a 1 and its proteolytic cleavage products was investigated in detail. Full-length Amb a 1 as well as Amb a 1α and β were expressed as recombinant His-tagged fusion proteins. All Amb a 1 molecules were compared in terms of IgE- and T-cell reactivity.

2. Methods

2.1. Patients and sera

Sera from patients with ragweed-pollen allergy as defined by clinical history, positive skin prick test (wheel diameter ≥3 mm), and IgE to ragweed pollen (CAP/RAST ≥ 3) were obtained from Canada (Dr. Alain Didierlaurent, Research Laboratory, Stallergenes S.A., Antony, France), Italy (Dr. Riccardo Asero, Ambulatorio di Allergologia, Clinica San Carlo, Paderno Dugnano, Italy), and Austria (Dr. Christof Ebner, Allergieambulatorium Reumannplatz, Vienna, Austria) and were stored at −20 °C. For T-cell studies heparinized peripheral blood was obtained from Austrian ragweed-pollen-allergic patients with approval by the ethics committee of the Medical University of Vienna (EK-No. 497/2005). Informed written consent was obtained from all subjects included in the study.

2.2. Purified nAmb a 1 and rabbit anti-sera

Natural Amb a 1 (nAmb a 1) as well as sera from rabbits immunized with nAmb a 1 were kindly provided by Prof. Te Piao King (Rockefeller University, NY, USA). NAmb a 1 was purified from ragweed-pollen extract as previously described (King et al., 1964, 1967).

2.3. N-terminal sequence analysis

NAmb a 1 was separated by SDS-PAGE and electroblotted onto polyvinyl difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Bands corresponding to Amb a 1 and its fragments were excised, and proteins were eluted by incubation in aqueous 40% (v/v) acetonitrile and 30% (v/v) trifluoroacetic acid. Samples were dried, resuspended in water, and sequenced with the HP G1005A protein sequencing system (Agilent Technologies).

2.4. Mass spectrometry

0.7 μg of purified nAmb a 1 protein solution in the presence of 100 mM DTT and 0.5 μl of a sinapinic acid matrix were dissolved in a saturated solution of 50% (v/v) acetonitrile and 0.1% (v/v) trifluoracetic acid, mixed, and applied to the target slide. Samples were analyzed with the Kompact MALDI-TOF IV mass spectrometer (Shimadzu) in the linear flight mode.

2.5. Cloning, expression, and purification of rAmb a 1, Amb a 1α and β

A ragweed-pollen cDNA library was constructed in the lambda ZAP II vector (Stratagene, La Jolla, CA, USA) as previously described (Wopfner et al., 2008b). Purified rabbit anti-Amb a 1-antibodies were used to screen the cDNA library according to the manufacturer's instructions. cDNA corresponding to isoform Amb a 1.3 was constructed into the vector pHis-parallel2 (Sheffield et al., 1999). Sequence was truncated at the 5′ end by 75 nucleotides coding for the putative signal peptide. The complete coding sequence of Amb a 1.3 and Amb a 1α, and β were modified by PCR by adding an NcoI site at the 5′ end and an XhoI site at the 3′ end (restrictions sites are underlined). Constructs rAmb a 1α and rAmb a 1β were amplified using rAmb a 1.3 as template and were mainly designed according to naturally processed chains, but considering the prevalent T-cell epitopes (Jahn-Schmid et al., manuscript in preparation), as rAmb a 1α: 520–1194 and rAmb a 1β: 76–519, respectively (see also Section 3.4). The following primers were used: for rAmb a 1.3 5′GAGACCATGGCCGAAGGGGTCGGAGAAATCTTAC3′ (Rag-Nco-fw) and 5′GAGACTCGAGTTAGCAAGGTGCTCCAGGACGGC3′ (Rag-Xho-rv), for rAmb a 1α: R-αII-Nco-fw 5'-GAGACCATGGTGCTTCCAGGAGGCATG-3' and Rag-Xho-rv, and for Amb a 1β amplification: Rag-Nco-fw and R-βII-Xho-rv 5'-GAGACTCGAGCTATTTAACATCATGGATATTTATG-3'.

Protein expression in *Escherichia coli* was performed as previously described (Wopfner et al., 2008b). Recombinant Amb a 1 molecules were purified as 6× His-tagged fusion protein from inclusion bodies by immobilized metal affinity chromatography. Pure fractions containing rAmb a 1.3, rAmb a 1α or β were pooled and various protocols for refolding were performed.

2.6. Homology modeling of Amb a 1

The Amb a 1 protein sequence (GI:166443) was submitted to profile sequence searches with the FFAS server (http://ffas.ljcrf.edu). The protein structure with highest scoring alignment from FFAS search, Jun a 1 (PDB-ID: 1pxz, residues 47–346, FFAS score of −93.1 sequence identity of 47%) was used as a template for homology modeling with the SCWRL-Server (http://www1.jcsg.org/scripts/prod/scwrl). Models were manually inspected, analyzed, and figures were prepared using Pymol (http://pyml.sourceforge.net, DeLano Scientific, Palo Alto, CA, USA).

2.7. SDS-PAGE and immunoblot analysis

NAmb a 1, rAmb a 1, rAmb a 1α, and β were analyzed by SDS-PAGE and purified proteins were visualized by staining with Coomassie Brilliant Blue R-250. For immunoblot analysis, the proteins were electroblotted onto nitrocellulose membranes (Protran, Schleicher and Schuell, Dassel, Germany) and incubated with sera from ragweed-allergic individuals as described before (Wopfner et al., 2008a).

2.8. Enzyme-linked immunosorbent assay

Maxisorp plates (Nagle Nunc, Rochester, NY) were coated with allergen (200 ng/well in 50 μl of PBS) overnight at 4 °C. Plates were blocked with Tris-buffered saline (pH 7.4), 0.05% Tween, and 1% BSA and incubated with patients' sera diluted 1:5 for 2 h. Bound IgE was