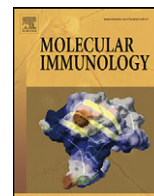




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The alpha and beta subchain of Amb a 1, the major ragweed-pollen allergen show divergent reactivity at the IgE and T-cell level

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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 proteolytic 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 1 α and Amb a 1 β , 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 1 α (aa 181–396) and Amb a 1 β (aa 26–180) were revealed. Amb a 1 β contained important IgE epitopes, whereas Amb a 1 α 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 1 α behaves as hypoallergen with reduced IgE binding but preservation of the major T-cell reactivity. In addition, recombinant Amb a 1 α can be easily purified to homogeneity in large quantity and therefore represents an ideal candidate for specific immunotherapy.

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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 1 α 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 polyvinylidene 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) trifluoroacetic 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 1 α 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 1 α , 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 1 α and rAmb a 1 β 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 1 α : 520–1194 and rAmb a 1 β : 76–519, respectively (see also Section 3.4). The following primers were used: for rAmb a 1.3 5'-GAGACCATGGCCGAAGGGTCCGAGAAATCTTAC3' (Rag-Nco-fw) and 5'-GAGACTCGAGTTAGCAAGGTGCTCCAGGACGGC3' (Rag-Xho-rv), for rAmb a 1 α : R- α II-Nco-fw 5'-GAGACCATGGTCTCCAGGAGGCGATG-3' and Rag-Xho-rv, and for Amb a 1 β amplification: Rag-Nco-fw and R- β II-Xho-rv 5'-GAGACTCGAGCTATTTAATCATGTGATATTTATG-3'.

Protein expression in *Escherichia coli* was performed as previously described (Wopfner et al., 2008b). Recombinant Amb a 1 molecules were purified as 6 $\times$ His-tagged fusion protein from inclusion bodies by immobilized metal affinity chromatography. Pure fractions containing rAmb a 1.3, rAmb a 1 α 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://pymol.sourceforge.net>, DeLano Scientific, Palo Alto, CA, USA).

2.7. SDS-PAGE and immunoblot analysis

NAmb a 1, rAmb a 1, rAmb a 1 α , 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

detected with alkaline phosphatase-conjugated monoclonal anti-human IgE antibodies (BD Biosciences, Franklin Lakes, NJ).

2.9. Mediator release assays

Rat basophil degranulation assays were performed as previously described (Vogel et al., 2005). Briefly, rat basophilic leukemia (RBL)-2H3 cells transfected with human high-affinity IgE receptor (FcεRI) were passively sensitized with serum IgE from patients allergic to Amb a 1. Degranulation was triggered by the addition of serial dilutions of nAmb a 1, rAmb a 1, rAmb a 1α, and β. Release of β-hexosaminidase into the supernatant was measured by means of enzymatic cleavage of the fluorogenic substrate 4-methyl umbelliferyl-N-acetyl-b-glucosaminide and expressed as the percentage of total enzyme content obtained by lysing the cells with Triton X-100.

2.10. T-cell response to Amb a 1 variants

Amb a 1-specific T-cell lines (TCL) were established from PBMC as described previously (Jahn-Schmid et al., 2002) with minor mod-

ification. Ragweed-pollen extract was used for the initial primary stimulation. For proliferation assays, TCL (2×10^4) were stimulated with nAmb a 1, rAmb a 1 ($10 \mu\text{g/ml}$ if not indicated otherwise), or equimolar concentrations of rAmb a 1α or rAmb a 1β in the presence of autologous irradiated (60 Gy) PBMC. T-cell proliferation was measured by [^3H]-thymidine uptake as described (Jahn-Schmid et al., 2002). Delta counts per minute (dpm) were calculated as mean cpm of cultures with stimulant – cpm of cultures without stimulant.

3. Results

3.1. Characterization of nAmb a 1 processing

SDS-PAGE analysis of purified nAmb a 1 revealed that the protein is cleaved into two chains, an approximately 26 kDa α-chain (nAmb a 1α) and a 15 kDa β-chain (nAmb a 1β) (Fig. 1A). To determine the exact cleavage site, purified nAmb a 1 was analyzed by Maldi-TOF mass spectrometry and Edman-degradation. Table 1 summarizes the results obtained by N-terminal sequencing. Briefly, taking isoform Amb a 1.1 as template for all calculations, cleavage of nAmb

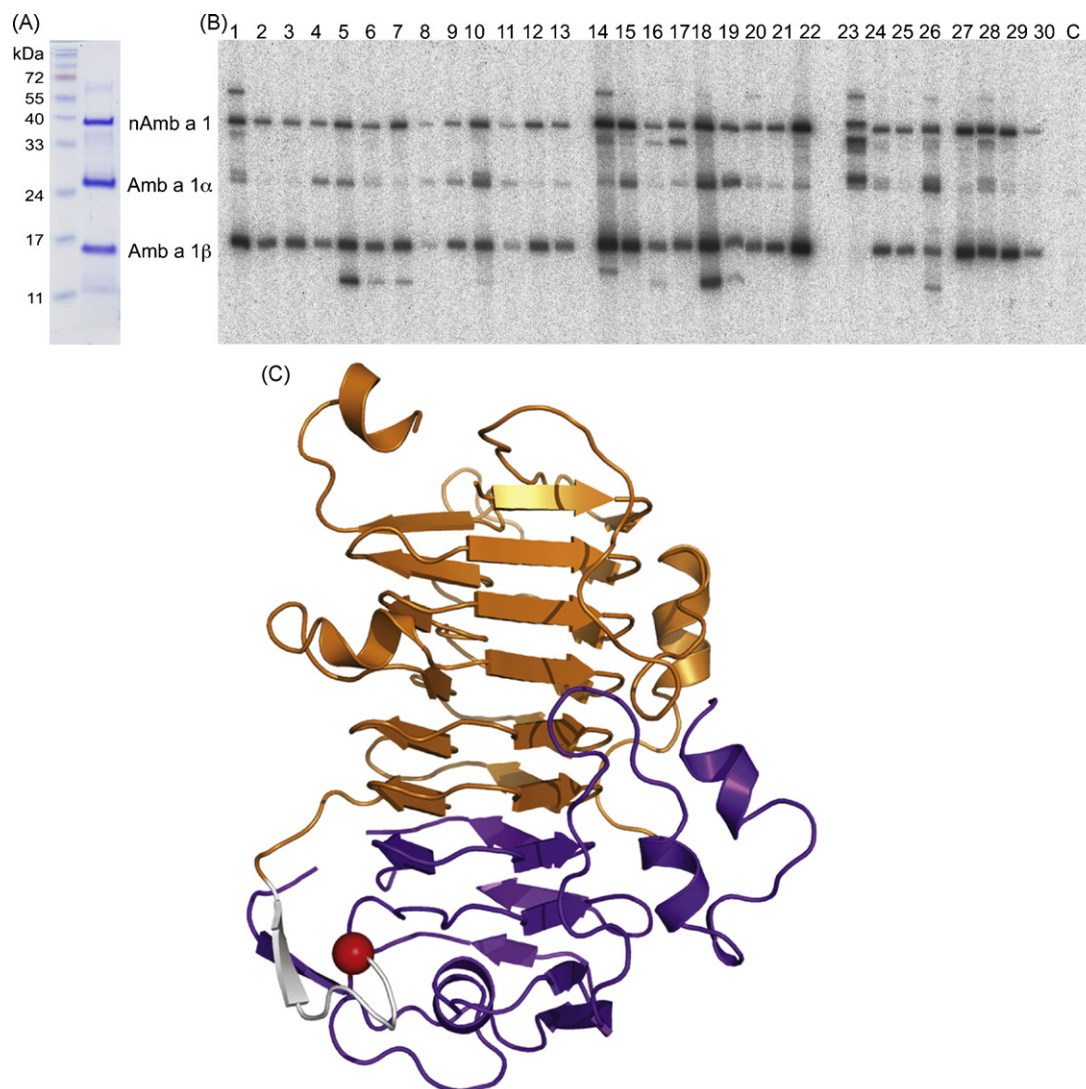


Fig. 1. Characterization of natural Amb a 1. (A) Coomassie stained SDS-PAGE of purified nAmb a 1. The bands corresponding to unprocessed nAmb a 1 at 38 kDa, Amb a 1α and Amb a 1β at 26 kDa and 15 kDa, respectively. (B) IgE-binding activity to nAmb a 1. Sera from ragweed-allergic patients (lanes 1–30) were tested in IgE immunoblots. Ragweed-pollen-sensitized patients were recruited in Italy (lanes 1–13), Canada (lanes 14–22) and Austria (lanes 23–30). Lane C indicates buffer control. (C) Amb a 1 homology model based on the crystal structure of Jun a 1 (PDB-ID: 1pxz). Residues are shown in ribbon representation. The region for Amb a 1β is blue and for Amb a 1α orange. Missing residues 181–190 are displayed in grey. The cleavage site of nAmb a 1 at residue K180 is depicted as a red sphere. (For interpretation of the references to color information in the figure, the reader is referred to the web version of the article.)

Table 1
N-terminal sequences of (A) full-length nAmb a 1, (B) nAmb a 1 α , and (C) nAmb a 1 β .

(A)	
	26 60
nAmb a 1	TSGAYNIIDGxxRKA...
Amb a 1.1	AEDLQEILP-VNETRR-LTTS
Amb a 1.2	AEDVVEFLPSANETRRSLKACEAHNIIDKCWRCKA...
Amb a 1.3	AEGVGEILPSVNETR-SLQACEAYNIIDKCWRCKA...
Amb a 1.4	AEDLQQLPSANETR-SLTTCGTYNIIDGCWRGKA...

(B)	
	192 206
nAmb a 1 α	APRDGDDISIIGSSQ...
nAmb a 1 α	APSAGSAIDAIGSSQ...
Amb a 1.1	AGSDGDAISISGSSQ...
Amb a 1.2	QQSDGDAINVAGSSQ...
Amb a 1.3	QASDGDITINVAGSSQ...
Amb a 1.4	KGSDGDAIGISGGSQ...

(C)	
	26 61
nAmb a 1 β	TSGAYNIIDGCxRGKAD...
Amb a 1.1	AEDLQEILP-VNETRR-LTTS
Amb a 1.2	AEDVVEFLPSANETRRSLKACEAHNIIDKCWRCKAD...
Amb a 1.3	AEGVGEILPSVNETR-SLQACEAYNIIDKCWRGKAD...
Amb a 1.4	AEDLQQLPSANETR-SLTTCGTYNIIDGCWRGKAD...

Sequences were determined by Edman-degradation. Alignment of deduced amino acid sequences of Amb a 1 isoforms (Amb a 1.1: accession P27759; Amb a 1.2: accession P27760, Amb a 1.3: accession P27761; and Amb a 1.4: accession P28744) are shown. The signal peptide sequence (residues 1–25) was not included. Differences within the four isoforms are shown in bold; gaps are indicated with a solid line. Residues that could not be clearly identified by N-terminal sequencing are marked with an x.

a 1 occurred at residue K180. Exact mapping of the Amb a 1 fragments revealed a complex proteolytic processing which includes removal of the signal peptide (residues 1–26), a peptide at the N-terminus of nAmb a 1 β (residues 27–43), and a peptide between the two chains (residues 180–189). Neither of the peptides was detectable and had been most likely degraded. nAmb a 1 α contains residues 189–396 and corresponding measured molecular weights of 21,808.54/22,323.24Da, respectively. A mixture of various isoforms could explain two peaks for Amb a 1 α by mass spec analysis. nAmb a 1 β contains residues 43–180 and a corresponding measured molecular weight of 15,086.31 Da. Mass analysis of full-length nAmb a 1 revealed a molecular weight of 37,832.14 and that the protein does not possess additional post-translational modifications (Table 2).

3.2. Immunological characterization of nAmb a 1

In order to study IgE binding, sera derived from Italian, Canadian, and Austrian ragweed-pollen-allergic patients were pre-selected in immunoblots using crude ragweed-pollen extract. According to immunoblots patients' were classified as Amb a 1 allergic, if IgE reactivity to protein/s in the range of 38 kDa was observed. All

selected sera reacted with the full-length molecule of purified Amb a 1 at 38 kDa and also strongly recognized nAmb a 1 β (97%) at 15 kDa. In contrast, nAmb a 1 α at 26 kDa was only weakly recognized by 66% of tested sera (Fig. 1B), indicating that the major IgE epitopes are located in the N-terminus of Amb a 1.

3.3. Amb a 1 homology modeling and sequence analysis

Modeling of Amb a 1 was performed to demonstrate the location of Amb a 1 α and Amb a 1 β within the three-dimensional protein structure, including cleavage sites and proteolytical removed peptides (Fig. 1C). The ribbon model clearly demonstrated that the cleavage site is located in a loop region, well accessible for proteases. Sequence searches with Amb a 1 (GI:166443) identified Jun a 1, the major allergen from cedar pollen, as the closest homologue with known structure. Jun a 1 is a member of the pectate lyases family of allergens and provides a reliable homology model since it has a sequence identity of 47% with a FFAS score = -93.1 to Amb a 1 (Jaroszewski et al., 2005). Amb a 1 contains two disulfide bonds between C54–C71 and C211–C235 and a probable one between C391 and C397. Amb a 1 also contains the highly conserved sequence motifs 207-WiDH-210 and 272-RXPXXR-277, which have been shown to be required for pectolytic activity (Midoro-Horiuti et al., 2003). Whether, Amb a 1 represents an active pectate lyase remains to be determined.

3.4. Characterization of rAmb a 1, rAmb a 1 α , and rAmb a 1 β

To obtain recombinant Amb a 1, a ragweed-pollen cDNA library was established and screened with a polyclonal rabbit anti-Amb a 1 serum. Positive clones were sequenced and Blast searches revealed that one of the isolated clones represented the 397-residue protein Amb a 1.3 (GenBank Accession C53240), with two amino acid substitutions (F389L, H392R). Subsequently, rAmb a 1 (residues 26–397), rAmb a 1 α (residues 174–397) and rAmb a 1 β (residues 26–173) were produced in *E. coli* as 6 $\times$ His-tagged fusion proteins, and purified to homogeneity (Fig. 2A). Recombinant Amb a 1 α (224 residues) and β (148 residues) were mainly designed according to naturally processed Amb a 1, with minor modification. Eight amino acids from the C-terminus of Amb a 1 β were transferred to the N-terminus of Amb a 1 α to maintain an important T-cell activating region. All proteins were expressed in inclusion bodies, extracted and subjected to conditions that should allow refolding. RAmb a 1 also showed proteolytic cleavage during expression and purification, resulting in two fragments similar in size to the α - and β -chain of nAmb a 1. Interestingly, the cleavage site of rAmb a 1.3 was found at residue 156 and not at 181 as in nAmb a 1, most likely due to different proteases present in plants and bacteria. Further, it is worth noting that the expression and refolding of Amb a 1 α resulted in yields up to 100-times higher compared to full-length rAmb a 1 or rAmb a 1 β , which had proven extremely problematic to produce.

3.5. IgE binding of rAmb a 1, rAmb a 1 α , and rAmb a 1 β

As shown above, nAmb a 1 α shows low IgE reactivity whereas nAmb a 1 β contains most of the IgE epitopes of full-length nAmb

Table 2
Mass spectrometry analysis of purified nAmb a 1.

	Unprocessed Amb a 1		Amb a 1 α		Amb a 1 β	
	Calculated	Measured	Calculated	Measured	Calculated	Measured
nAmb a 1		37,832.14		21,808.54 22,323.24		15,086.31
Amb a 1.1	37,864.43		21,999.50		15,017.04	
Amb a 1.2	38,625.71		22,425.10		15,268.56	
Amb a 1.3	38,255.30		22,036.77		15,286.49	
Amb a 1.4	38,008.93		21,953.62		15,130.29	

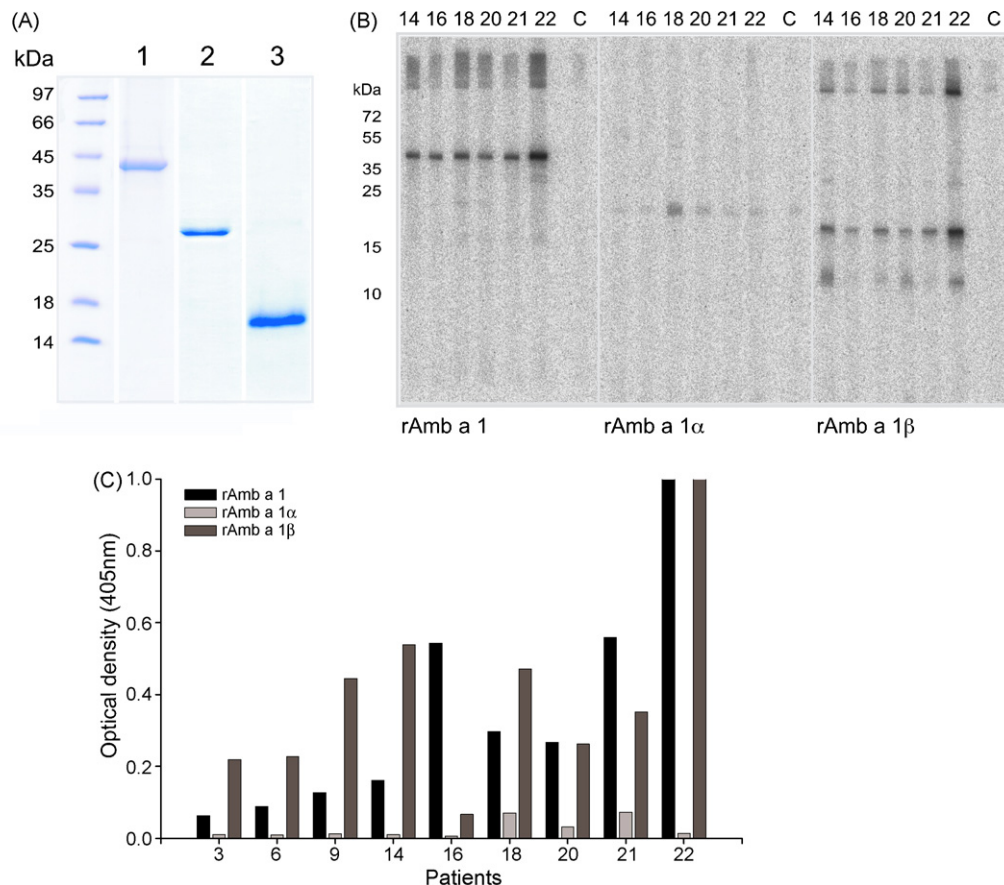


Fig. 2. Immunological characterization of rAmb a 1.3, rAmb a 1 α and - β . (A) Purified His-tagged rAmb a 1.3 (lane 1), rAmb a 1 α (lane 2) and β (lane 3) were separated by SDS-PAGE followed by Coomassie Brilliant Blue staining. (B) IgE binding properties of purified recombinant proteins were evaluated using six pre-selected Canadian patients. rAmb a 1 and rAmb a 1 β showed comparable IgE reactivity, whereas rAmb a 1 α showed no IgE binding. Lane C indicates buffer control. (C) IgE reactivity under native conditions. ELISA experiments were performed using three Italian (patients 3, 6 and 9) and six Canadian sera (patients 14–22). rAmb a 1 and rAmb a 1 β did only show weak IgE reactivity, rAmb a 1 α did not show any or only very little IgE binding.

a 1. In immunoblot experiments rAmb a 1 and rAmb a 1 β showed the same ability to bind human IgE compared to the natural counterparts (Fig. 2B). However, in ELISA experiments under native conditions, lower IgE reactivity was observed for rAmb a 1 and rAmb a 1 β for most of the tested patients (Fig. 2C). rAmb a 1 α showed low/no IgE-binding activity in both *in vitro* assays, independent from assay conditions (Fig. 2B and C). Using ELISA, rAmb a 1 α showed less than 10% of the IgE binding to nAmb a 1 (data not shown). Similar to rAmb a 1, rAmb a 1 β reacted with patients' IgE only after reduction and heat treatment in immunoblots, but not in ELISA.

3.6. Allergenic activity of Amb a 1 molecules

To evaluate the allergenic activity of all Amb a 1 molecules presented above, we performed mediator release assays using rat basophilic leukemia (RBL) cells transfected with Fc ϵ RI, the high-affinity receptor for human IgE (Vogel et al., 2005). RBL cells were sensitized by patients' sera and challenged with increasing concentrations of nAmb a 1, rAmb a 1, and rAmb a 1 α , and rAmb a 1 β . We found that all recombinant Amb a 1-related proteins exhibited a low capacity to induce mediator release. In general, the release curves for nAmb a 1 and rAmb a 1 were completely different. nAmb a 1 induced the highest release values, whereas rAmb a 1 triggered comparable responses at 1000–10,000-fold higher concentrations (Fig. 3). rAmb a 1 α and β were not able to induce mediator release, even at 10,000-fold higher concentration (Fig. 3).

3.7. T-cell reactivity to nAmb a 1, rAmb a 1, rAmb a 1 α , and rAmb a 1 β

The T-cell response to nAmb a 1, rAmb a 1 and rAmb a 1 α and rAmb a 1 β was analyzed in oligoclonal, Amb a 1-specific TCL derived from ragweed-pollen-allergic donors. nAmb a 1 and rAmb a 1 induced very similar T-cell proliferations in 13 TCLs derived from 12 different donors (Fig. 4A). The T-cell responses to the shorter proteins of rAmb a 1 α and rAmb a 1 β were compared to rAmb a 1 at equimolar protein concentrations in 25 TCL from 17 different patients. A titration curve of one representative TCL is shown in Fig. 4B. Overall, stimulation by rAmb a 1 α induced a mean of 80% of the proliferation obtained in response to rAmb a 1, which was significantly higher than the proliferative response to rAmb a 1 β reaching a mean of 37% (Fig. 4C). Together, rAmb a 1 stimulated comparable T-cell responses as nAmb a 1 and the T-cell reactivity was largely directed to rAmb a 1 α .

4. Discussion

NAmb a 1 undergoes limited proteolysis during purification and it is cleaved into a 26 kDa α -chain and a 15 kDa β -chain, which are non-covalently associated. So far, it was demonstrated that this Amb a 1 two-chain form seems to be immunologically indistinguishable from the full-length molecule (King et al., 1981).

In the present study, for the first time we show divergent immunologic properties of the two Amb a 1 chains. Independent of their origin, the vast majority of sera derived from ragweed-allergic

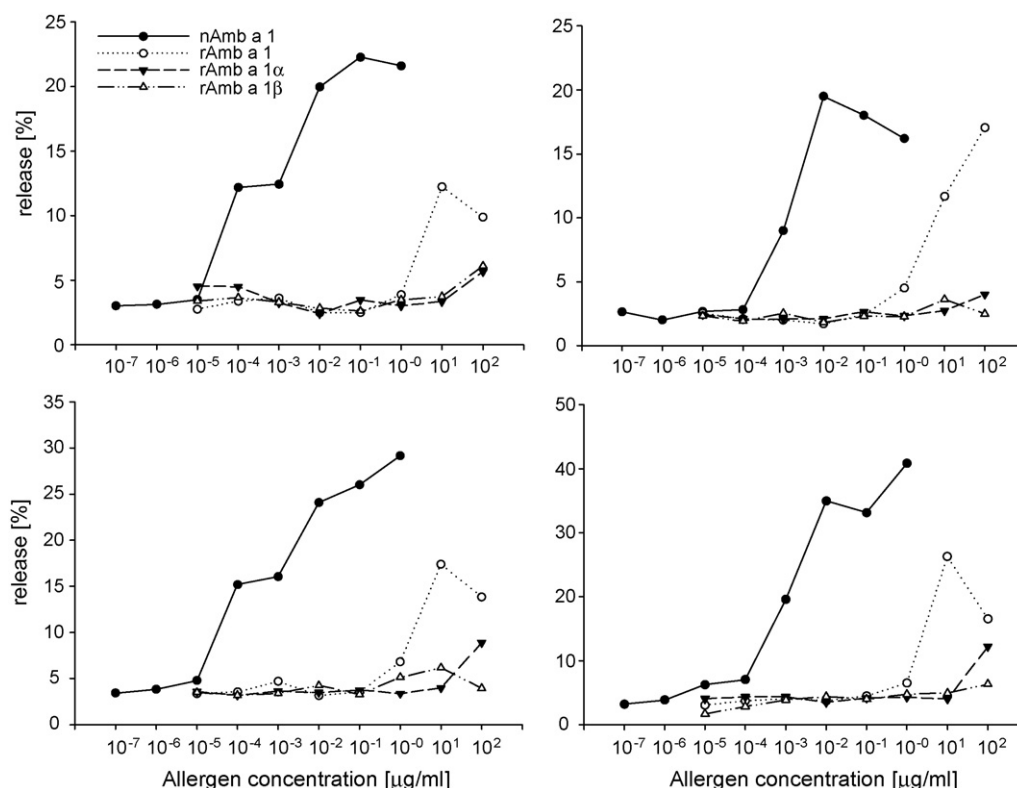


Fig. 3. Mediator release capacity of Amb a 1 allergens. Induction of β -hexosaminidase release from RBL-2H3 cells expressing the human Fc ϵ RI. Rat basophil leukemia cells were passively sensitized with IgE antibodies from four ragweed-sensitized patients. Mediator release was triggered by serial dilutions of nAmb a 1 (10^{-7} – 10^0 μ g/ml) and rAmb a 1, Amb a 1 α or rAmb a 1 β (10^{-5} – 10^2 μ g/ml). Degranulation was measured by assaying β -hexosaminidase in the supernatant and shown as percentage of degranulation achieved by total mediator release induced by cell lysis.

patients from Canada, Italy and Austria reacted with nAmb a 1 β . In contrast, nAmb a 1 α showed only weak IgE reactivity.

IgE reactivity of rAmb a 1, however, varied depending on the assay used. In non-denaturing assays (e.g., dot blot, ELISA) rAmb

a 1 bound human IgE weakly, while IgE binding of rAmb a 1 in immunoblots was similar to the natural protein. In addition, native rAmb a 1 was not able to trigger IgE-mediated histamin release from effector cells. Since rAmb a 1 is expressed exclusively in inclusion

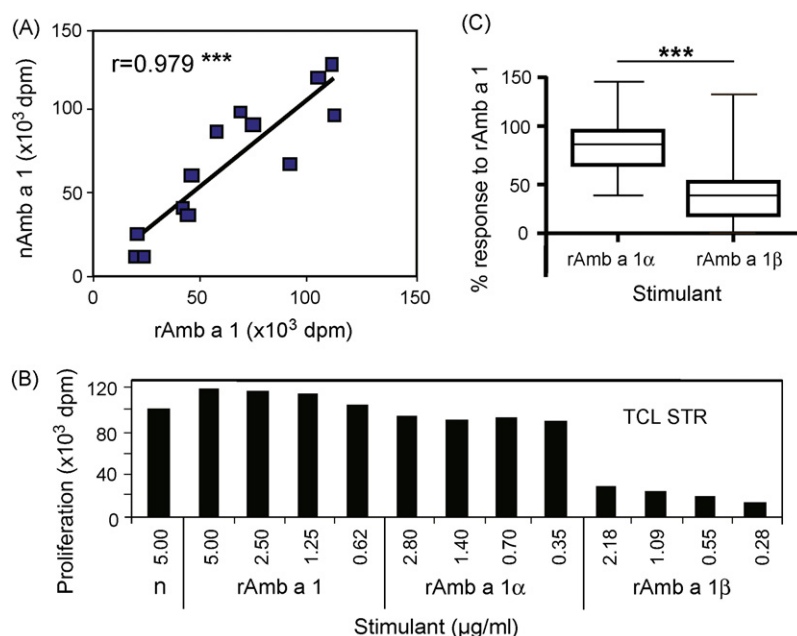


Fig. 4. T-cell responses to Amb a 1 variants. Amb a 1-specific TCL were established from PBMC of ragweed-allergic donors. Proliferation in response to different Amb a 1 proteins was measured by ^{3} H-thymidin-uptake after 48 h. (A) Comparison of 13 TCL stimulated with 10 μ g/ml nAmb a 1 or rAmb a 1. (B) Concentration-dependent stimulation with rAmb a 1 and equimolar amounts of rAmb a 1 α or rAmb a 1 β . One representative experiment out of 12. (C) Proliferative response of 25 TCL to rAmb a 1, Amb a 1 α and rAmb a 1 β at optimum concentrations. Box plots are shown. The significance of the difference ($p < 0.001$) was calculated by Wilcoxon Signed Ranks test. dpm: cpm of stimulated cultures – cpm of medium control cultures. r: Pearson correlation coefficient (significance: *** $p < 0.001$).

bodies and requires a refolding protocol for purification, incomplete or incorrect refolding may explain the lack of IgE reactivity to the native protein. Only after rigid denaturation (SDS-PAGE) and supposed refolding on nitrocellulose, rAmb a 1 seems to take the natural protein structure, which could explain binding to human IgE in immunoblot experiments.

Nevertheless, since T-cell responses are independent of protein conformation, rAmb a 1.3 could be successfully used for proliferation assays (Fig. 4A). Natural and rAmb a 1.3 induced similar T-cell responses regarding proliferation as well as cytokine production (data not shown). This finding indicated that, although nAmb a 1 consists of four different isoforms with sequence identities of 80% (Griffith et al., 1991), rAmb a 1.3 is recognized by the majority of Amb a 1-reactive T cells. A high cross-reactivity between Amb a 1.1 and Amb a 1.3 at the T-cell level had been shown also by Bond et al. (1991). Therefore, rAmb a 1.3 could be considered for application in a T-cell-based specific immunotherapy.

Our findings prompted us to investigate the possibility to separately produce the subchains of Amb a 1 in *E. coli* for application in specific immunotherapy. rAmb a 1 α and rAmb a 1 β were mainly designed according to naturally processed Amb a 1. rAmb a 1 α was designed to contain the most important T-cell epitopes. Therefore, eight residues from the C-terminus of Amb a 1 β were added to the original rAmb a 1 α sequence. Modified proteins were expressed in inclusion bodies, purified and refolded. The protein yields for rAmb a 1 α was 10-fold higher than for rAmb a 1.3, and >100-fold higher as compared to rAmb a 1 β , amounting to 16 mg refolded pure Amb a 1 α /11 fermentation culture. Furthermore, purified rAmb a 1 α did not show any tendency of aggregation, which had turned out to be a crucial problem when expressing the full-length rAmb a 1.3 or rAmb a 1 β . Interestingly, even though rAmb a 1 β was slightly modified and contained eight C-terminal amino acids less than the naturally processed Amb a 1 β , it showed similar IgE-related behaviour as full-length rAmb a 1.3: (i) low IgE reactivity under native conditions, (ii) comparable IgE binding to nAmb a 1 under denaturing conditions and (iii) no biological activity with regard to mediator release. In striking contrast, rAmb a 1 α did not show IgE reactivity, neither under reducing nor under native conditions. It appears that all recombinant forms of Amb a 1, the full-length form, as well as both fragments, are hypoallergenic in terms of IgE binding.

Amb a 1 is the main target for specific immunotherapy of ragweed-pollen allergy, since 95% of the ragweed-sensitized patients react to nAmb a 1 in skin tests and show high IgE antibody titers. The benefit of SIT has been confirmed in many clinical studies using either pollen extracts (Durham et al., 1999), and more recently also recombinant wild-type (Jutel et al., 2005) and genetically modified (Niederberger et al., 2004) allergens. Recently performed clinical studies using purified nAmb a 1 coupled to immunostimulatory DNA sequences (ISS) demonstrated efficacy, even though the patients were solely immunized with the major allergen (Creticos and Lichtenstein, 2003; Creticos et al., 2006; Higgins et al., 2006).

Taken together, we could demonstrate that modified rAmb a 1 α produced in *E. coli* represents a hypoallergen, i.e. a molecule with reduced allergenic activity and retained T-cell-based immunogenicity. The low IgE-binding activity and reduced capacity to induce release of inflammatory mediators combined with high potency to activate Amb a 1-specific T cells is expected to minimize IgE-mediated side effects during SIT without compromising efficacy. The reason for the lack of IgE reactivity in ELISA or basophil degranulation assays is unclear but most probably is due to improper folding of rAmb a 1 produced in bacteria. We are currently investigating this problem with detailed mass spec analyses and are evaluating plant expression systems for the production of rAmb a 1. Nevertheless, rAmb a 1 shows all characteristics of a hypoallergen and is therefore a promising candidate for T-cell-based specific immunotherapy. Furthermore, in particular Amb a 1 α , which com-

pared to rAmb a 1 is much more suitable for large-scale production and can be easily purified to homogeneity, would represent an ideal candidate molecule for specific immunotherapy that could replace nAmb a 1 in specific immunotherapy of ragweed-pollen allergy.

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