

ORIGINAL PAPER

Immunologic analysis of monoclonal and immunoglobulin E antibody epitopes on natural and recombinant Amb a 1

N. Wopfner*, R. Bauer*, J. Thalhamer*, F. Ferreira* and M. Chapman†

*Christian Doppler Laboratory for Allergy Diagnosis and Therapy, Department of Molecular Biology, University of Salzburg, Austria and †Indoor Biotechnologies Inc., Charlottesville, Virginia, United States

Clinical and Experimental Allergy

Summary

Background Amb a 1 is the major allergen from ragweed pollen and more than 90% of ragweed-allergic patients react with this protein. Although Amb a 1 was cloned and sequenced in 1991, little is known of the specificity of anti-Amb a 1 antibodies or of the immunologic properties of the recombinant allergen.

Objective To compare binding of monoclonal antibodies (mAb) and IgE antibodies to purified natural Amb a 1 (nAmb a 1) and recombinant Amb a 1 (rAmb a 1).

Methods Binding of a panel of anti-Amb a 1 mAb and IgE antibodies to nAmb a 1 or rAmb a 1 was compared by immunoblotting. Chimeric ELISA was used to measure specific IgE to these allergens using 89 ragweed-allergic sera from Austria, Italy, Canada and the United States.

Results The 8 mAb bound to a 38 kDa Amb a 1 band in ragweed pollen extract and a subset of 5 mAb also bound to the 26 kDa chain of nAmb a 1. A two-site ELISA was developed using a mAb pair, which was ~10-fold more sensitive to rAmb a 1. There was a significant correlation between IgE antibody binding to nAmb a 1 and rAmb a 1 ($n = 89$, $r = 0.79$, $P < 0.001$).

A subset of ~40% of patients showed greater reactivity to nAmb a 1 than to rAmb a 1.

Conclusions The data suggest that there is less reactivity of human IgE to rAmb a 1 compared with nAmb a 1. The development of more sensitive, quantitative ELISA for Amb a 1 will require the production of new mAb especially directed against nAmb a 1.

Keywords chimeric ELISA, IgE antibody, immunotherapy, monoclonal antibodies, natural Amb a 1, ragweed allergy, recombinant Amb a 1

Submitted 2 April 2007; revised 23 August 2007; accepted 21 September 2007

Correspondence:

Nicole Wopfner, Christian Doppler Laboratory for Allergy Diagnosis and Therapy, Department of Molecular Biology, University of Salzburg, Hellbrunnerstrasse 34, A-5020 Salzburg, Austria.
E-mail: nicole.wopfner@sbg.ac.at

Introduction

Besides grasses and birch pollen, the pollen of short ragweed (*Ambrosia artemisiifolia*) is arguably one of the most clinically important and is responsible for the majority of cases of seasonal allergic rhinitis, especially in the United States. Ragweed has its origin in North America and represents a major source of allergenic proteins in the United States. It has been suggested that the prevalence of IgE sensitization to ragweed is up to 50% in pollen-allergic individuals [1]. Ragweed is also an important source of pollinosis in Canada, Japan, Australia and parts of Europe. Distribution of ragweed in Europe started after the First World War and was initially limited to Hungary. Ragweed has since become established in three main regions: the Carpathian Basin; the Rhône

valley (France), Northern Italy; and parts of Austria [2]. In these areas, allergic sensitization to ragweed has steadily increased. Long-term observational data from Vienna, Austria, showed that among patients with respiratory allergy, specific IgE detection to ragweed pollen increased from 20% in the 1980s to about 30% at the end of the 1990s. These findings correlate well with a threefold elevated annual ragweed pollen load over the same time period [3]. Ragweed is also a plant of concern in global warming because higher levels of carbon dioxide (CO₂) increase pollen and Amb a 1 production [4, 5]. These data suggest that increases in atmospheric CO₂ could directly increase sensitization to ragweed pollen and the prevalence and/or severity of seasonal hayfever [4].

Amb a 1 is an acidic 38 kDa non-glycosylated protein with multiple isoelectric forms that belong to the

family of pectate lyase proteins [6–8]. In one study, more than 90% of ragweed-sensitive individuals reacted in skin tests and showed high IgE antibody titres to Amb a 1 [9]. Furthermore, up to 75% of the total serum IgE from ragweed-allergic patients is directed against this major allergen [10]. Natural Amb a 1 (nAmb a 1) undergoes proteolysis during purification and is cleaved into two non-covalently associated chains of 26 and 12 kDa [9, 11]. The two-chain form of the protein is antigenically indistinguishable from the full-length molecule [9].

Measurements of IgG and IgE antibody to nAmb a 1 were used to demonstrate the efficacy of conventional immunotherapy for ragweed in the classic clinical studies of Norman et al. in the 1960s and 1970s [12–16]. Amb a 1 was one of the first allergens to be cloned and sequenced over a decade ago [7, 17]. However, while other important pollen allergens (e.g. Bet v 1, Phl p 1, Phl p 5, Art v 1) have been produced as recombinant proteins and used to develop new vaccines, little work has been carried out on the expression of recombinant Amb a 1 (rAmb a 1) or on the immunologic reactivity of the recombinant protein [18, 19]. Expression of immuno-reactive rAmb a 1 is a pre-requisite for crystallographic studies and for structural modifications to develop new and improved forms of ragweed immunotherapy. Recently, rAmb a 1 has been expressed in *Escherichia coli* with an N-terminal His tag. This study compares the binding of monoclonal antibodies (mAb) raised against nAmb a 1 or rAmb a 1 to each of these allergens. We also compare IgE binding to nAmb a 1 and rAmb a 1 using a large panel of ragweed-allergic sera from North America and Europe. The results show a good correlation between IgE antibody binding to nAmb a 1 and rAmb a 1. Further improvements in the rAmb a 1 will enhance its immuno-reactivity and its use in allergy diagnostics and therapeutics.

Materials and methods

Pollen extracts and purified Amb a 1

Short ragweed pollen (*A. artemisiifolia*) (Allergon AB, Angelholm, Sweden) was extracted by suspending 150 mg of pollen in 1 mL of distilled water and shaking at room temperature for 15 min. After centrifugation at 14 000 g, the supernatant was collected and stored at -20°C until use. Commercial available skin prick test (SPT) solutions of short ragweed pollen were purchased from ALK-Abelló (Madrid, Spain), Greer (Lenoir, NC, USA) and Hollister–Stier (Spokane, WA, USA). nAmb a 1 was purified from ragweed pollen extract as described [6, 20]. Recombinant 6 $\times$ histidine-tagged Amb a 1.3 (rAmb a 1.3, swiss prot accession number: P27761) was obtained from Biomay AG (Vienna, Austria).

Immunization and monoclonal antibody production

For antibody production, pairs of 6–8-week-old BALB/c mice (Himberg, Austria) were inoculated (day 1) intraperitoneally (i.p.) with either 5 μg of nAmb a 1 or rAmb a 1 adsorbed to 100 μL alum (Serva, Heidelberg, Germany) in a total volume of 150 μL sterile $1 \times \text{PBS}$. Intraperitoneal boosters of 5 μg protein adsorbed to alum were given on day 14 and 21. Amb a 1-specific humoral immune response was monitored via ELISA. Once sufficient titres were detected, two final i.p. boosts of 5 μg protein (adsorbed to alum) were given one and four days before killing the mice to collect B cells for hybridoma production. Hybridoma fusion was carried out using BALB/c myeloma cell line P3X63Ag8.653 (ATCC, LGC Promochem, Wesel, Germany) and classical fusion protocols with minor modifications [21]. After fusion and recovery, hybridoma supernatants were screened using purified nAmb a 1 or rAmb a 1 as antigen. Amb a 1-specific antibodies were picked, subcloned in 96-well cell culture plates and isotyping of mAb from different hybridoma clones was performed. After a subsequent ELISA screening procedure, positive hybridoma clones originating from only one single cell were expanded. Eight different mAb clones were chosen to produce supernatants in large quantities. Those mAb clones were obtained by immunization either with purified nAmb a 1 (mAb B7) or with rAmb a 1 (mAb E2, C4, C6, C11, D4, E6, E10), subtype IgG1.

Human sera

A panel of sera from 89 patients allergic to ragweed pollen was selected according to clinical history, positive SPT and specific IgE concentration detected by means of commercial laboratory systems to ragweed pollen extract. Thirty-five sera from ragweed-allergic patients were from Canada (Dr Alain Didierlaurent, Research Laboratory, Stallergenes S. A., Antony, France), 15 from Italy (Dr Riccardo Asero, Ambulatorio di Allergologia, Clinica San Carlo, Paderno Dugnano, Italy), 31 from Austria (Dr Christof Ebner, AKH, Vienna, Austria) and eight from the United States (Dr Robert Hamilton, Johns Hopkins University, Baltimore, MD, USA). All patients gave their consent to enter the study. Sera were stored at -20°C .

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotting

Crude ragweed pollen extract (30 μg total protein), commercial SPT solutions (each 10 μL), nAmb a 1 (6 μg) and 6 $\times$ histidine-tagged rAmb a 1 (4 μg) were separated by denaturing SDS-PAGE and visualized by staining with Coomassie Brilliant Blue R-250 [22]. For immunoblot analysis of mAb, the proteins were electroblotted [23]

onto nitrocellulose membranes (Protran, Schleicher & Schuell, Dassel, Germany) and incubated with cell culture supernatants of monoclonal anti-Amb a 1 antibodies (mAb B7, E2, C4, C6, C11, D4, E6, E10) diluted 1:1000–1:12 000. After incubation, bound antibodies were detected with alkaline phosphatase-conjugated anti-mouse IgG (Dianova, Germany). For IgE immunoblots, sera from atopic individuals were diluted 1:10 in 50 mM sodium phosphate buffer, pH 7.4, containing 0.5% (v/v) Tween-20, 5% (w/v) bovine serum albumin (BSA) and 0.05% (w/v) sodium azide, and incubated with blotted ragweed pollen extracts as well as with rAmb a 1 or nAmb a 1 overnight at 4 °C. Bound IgE antibodies were detected using ¹²⁵I-rabbit anti-human IgE (MALT Allergy system, IBL GmbH, Hamburg, Germany) on a phosphorimager screen (Fujifilm BAS-1800 II, Tokyo, Japan).

Enzyme-linked immunosorbent assay for Amb a 1

For ELISA, antibody combinations were chosen based on test results obtained from either coating protein G-purified mAb clones C4, C6, E6 or E10 on maxisorp ELISA plates (NUNC, Roskilde, Denmark) and using biotinylated C4 as the detection antibody. Antibody was biotinylated using EZ-Link sulphy-NHS-LC-Biotin [sulphosuccinimidyl-6-(biotinamido) hexanoate] (Pierce, Rockford, IL, USA) following manufacturer's guidelines. Highest sensitivity in detection was achieved using the C4/E10 mAb clone combination. For Amb a 1-specific ELISA, plates were therefore coated with 2 µg/mL anti-Amb a 1 mAb E10 or C4 overnight at 4 °C. After washing, plates were blocked with 1% (w/v) BSA-PBS-Tween and incubated with serial two-fold dilutions of purified nAmb a 1 or rAmb a 1 (0.01–5 µg/mL) or with dilutions of commercial ragweed pollen extracts (1:50–1:25 600) for 1 h. For Amb a 1 detection, plates were washed and incubated with biotinylated anti-Amb a 1 mAb C4 diluted 1:1000 for 1 h followed by incubation with streptavidin-peroxidase (1:1000) (Sigma, St Louis, MO, USA). The reaction was developed using 1 mmol 2,2'-azino-bis (3 ethylbenzthiazoline-6 sulphonic acid) (Sigma) and H₂O₂ and optical density was measured at 405 nm.

To detect specific IgE, E10 was used as capture antibody followed by blocking and incubation with purified nAmb a 1 or rAmb a 1 (1 µg/mL), or with different commercial ragweed pollen extracts (1:100) for 1 h. After washing, the plates were incubated with 11 patients' sera diluted 1:4 for 1 h. Bound IgE-antibodies were detected using biotinylated goat anti-human IgE (1:4000 for 1 h) (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) followed by incubation with streptavidin-peroxidase (1:1000). The plates were developed as described above.

Quantification of immunoglobulin E antibody to natural Amb a 1 and recombinant Amb a 1

Specific IgE antibodies to nAmb a 1 and rAmb a 1 were measured using a chimeric ELISA, as previously described [24, 25]. Microtitre plates were coated with 2 µg/mL anti-Amb a 1 mAb E10 overnight at 4 °C. Plates were washed, blocked with 1% (w/v) BSA-PBS-Tween for 30 min and incubated with 1 µg/mL either nAmb a 1 or rAmb a 1 for 1 h. Plates were washed and incubated with sera diluted 1:2 and 1:10 for 1 h. Bound IgE was detected with biotinylated anti-human IgE and streptavidin-peroxidase, as described above. The assay was quantified using chimeric mouse/human IgE anti-Der p 2 mAb to form a control curve [25].

Results

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotting analyses of monoclonal antibody and immunoglobulin E antibody

Analyses of various ragweed pollen extracts, separated by SDS-PAGE and subsequent Coomassie staining demonstrated a similar protein pattern in all preparations (Fig. 1a). In all extracts, prominent bands migrating at about 38 kDa could be identified as Amb a 1 (Fig. 1a, lanes 3–6). Recombinant His-tagged Amb a 1 migrated at approximately 42 kDa, purified nAmb a 1 migrated at 38 kDa and specific degradation into two fragments of 12 and 26 kDa could be observed only with the nAmb a 1 (Fig. 1a). Analysis of Amb a 1-specific mAb by immunoblotting revealed that all mAb bound to nAmb a 1 in ragweed pollen extract, showing a double band at ~38 kDa. The mAb raised against nAmb a 1 (clone B7) reacted exclusively with nAmb a 1, whereas those raised against rAmb a 1 recognized both nAmb a 1 and rAmb a 1 (Fig. 1b). Three mAb (clones D4, E6, E10) only bound to the intact molecule at 38 kDa, whereas mAb B7, E2, C4, C6, C11 reacted both with the intact molecule and with the nAmb a 1-derived 26 kDa chain. None of the mAb reacted with the 12 kDa chain alone (Fig. 1b).

For IgE-binding study, sera were pre-selected in immunoblots using crude ragweed pollen extract. Patients were classified as Amb a 1 allergic if reactivity to a protein in the range of 38 kDa was observed. About 40 of those Amb a 1-allergic patients were used to investigate IgE binding to purified nAmb a 1 and rAmb a 1 by immunoblotting. Figure 2 shows a representative group of eight Italian sera and two Austrian patients who showed similar patterns of IgE reactivity when compared under reducing conditions on SDS-PAGE: a single band at ~38 kDa using recombinant Amb a 1 (Fig. 2a); and additional binding to proteolytic products

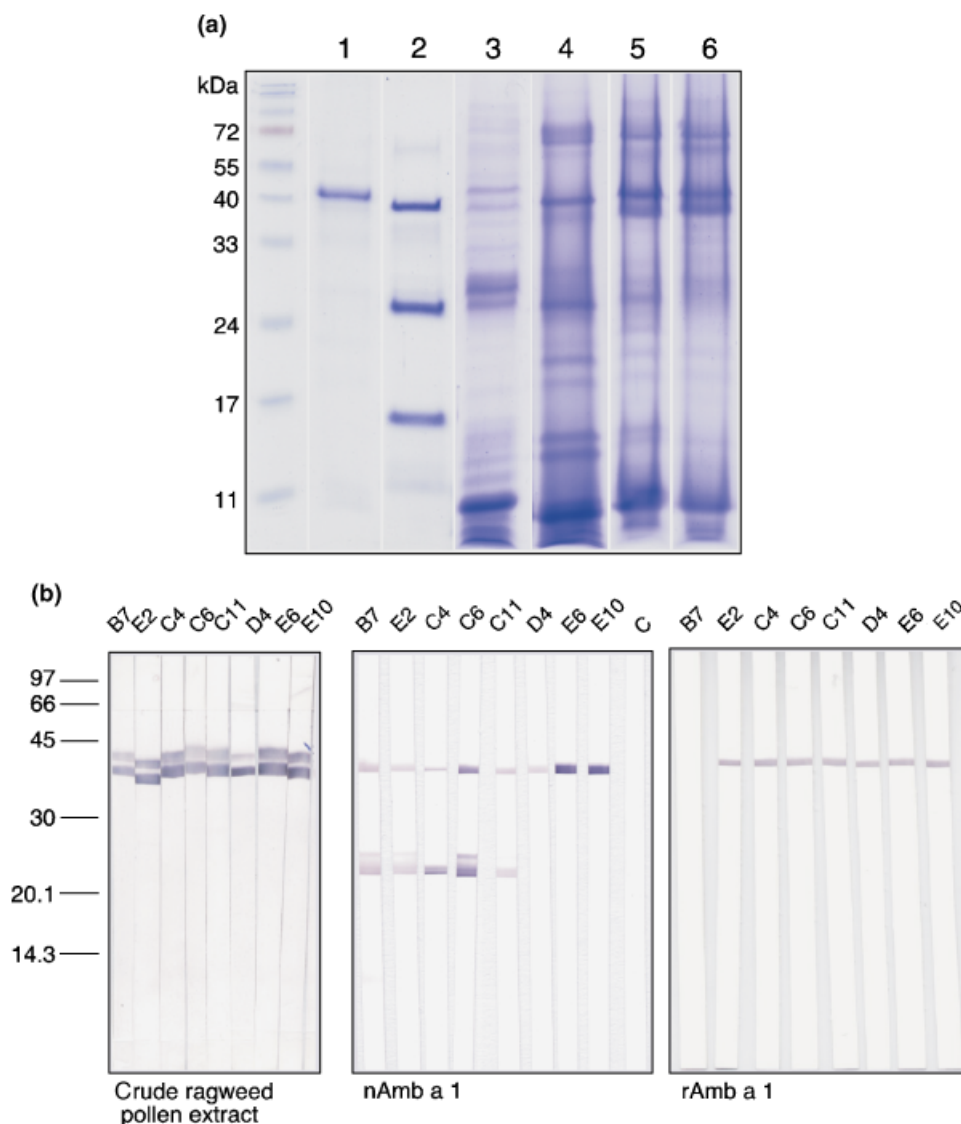


Fig. 1. Purified allergens, pollen extracts and immunoblotting analyses using Amb a 1 mAb. (a) Recombinant 6 × histidine-tagged (lane 1) and natural Amb a 1 (lane 2) as well as crude ragweed pollen extract (lane 3), and commercially available skin prick test solutions from ALK-Abelló (lane 4), Greer (lane 5) and Hollister-Stier (lane 6) were separated by SDS-PAGE followed by Coomassie Brilliant Blue staining. (b) Reactivity of eight Amb a 1-specific mAb (clones B7, E2, C4, C6, C11, D4, E6 and E10) with nitrocellulose-blotted crude ragweed pollen extract [sample corresponding to lane 3, (a)], natural and recombinant Amb a 1. Lane C indicates buffer control, leaving out the mAb. Molecular weight markers are indicated at the left. mAb, monoclonal antibodies.

of purified Amb a 1 (Fig. 2b). Testing these patients with commercially available extracts revealed comparable IgE reactivity (Fig. 2c–f). Interestingly, both mAb and human IgE recognized a double band in the range of 38 kDa, corresponding to Amb a 1 in ragweed extracts, which may be due to the presence of different isoforms in pollen. Nevertheless, while it looked as if the mAbs recognized the two apparent bands of Amb a 1 equally well, the patients' sera seemed to favour one form (exception: patient 4). These immunoblotting results suggested that IgE antibodies recognized both nAmb a 1 and rAmb a 1.

Two-site monoclonal antibody enzyme-linked immunosorbent assay for Amb a 1

The immunoblotting results also suggested that some of the mAb were directed against different epitopes. A two-site ELISA was developed using either mAb E10 or C4 as capture mAb and biotinylated mAb C4 antibody for detection. Also other mAb combinations have been tested for the suitability (capture mAb: E6, C6, and C4) but because the mAb E10 only bound to intact 38 kDa Amb a 1, whereas mAb C4 bound to the intact molecule and to the 26 kDa α -chain this combination was chosen. Both

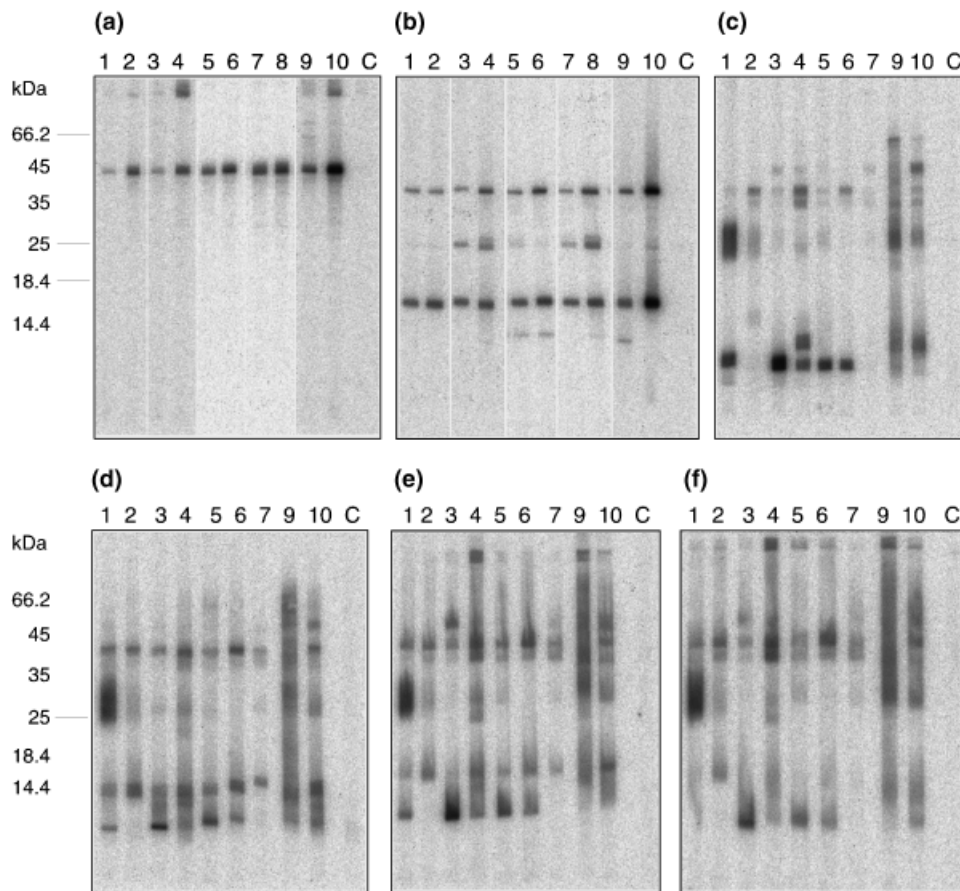


Fig. 2. IgE immunoblot analyses of purified allergens and ragweed extracts. IgE reactivity from eight Italian patients (lanes 1–8) and two Austrian patients (lanes 9 and 10) was compared with nitrocellulose-blotted recombinant 6 × histidine-tagged Amb a 1 (a), purified natural Amb a 1 (b), crude ragweed pollen extract (c), and skin prick test solutions from ALK-Abelló (d), Greer (e) and Hollister–Stier (f). Lane C indicates buffer control (leaving out the serum). Molecular weight markers are indicated at the left.

nAmb a 1 and rAmb a 1 reacted in this ELISA in a dose-dependent manner. However, there were significant differences in the slopes of the binding curves for the two forms of Amb a 1 and the ELISA was ~10-fold more sensitive to rAmb a 1 (Fig. 3a). Although purified nAmb a 1 reacted in the assay, it was not possible to detect Amb a 1 in any of the commercial or self-made ragweed pollen extracts (Fig. 3b).

Comparison of human immunoglobulin E antibody binding to natural and recombinant Amb a 1

The ELISA results suggested that one of the mAb that was raised against rAmb a 1 did not bind to nAmb a 1. As an alternative approach, a chimeric ELISA was established using IgE antibodies to Amb a 1 to investigate (i) whether nAmb a 1 in pollen extracts could be detected using mAb in combination with IgE antibodies (ab); and (ii) to directly compare IgE reactivity for nAmb a 1 and rAmb a 1. Using mAb E10 for allergen capture, and IgE antibodies in sera from ragweed-allergic patients ($n = 11$) for detection, it

was possible to detect Amb a 1 in commercial ragweed pollen extract from ALK, Greer and Hollister–Stier. The IgE ab also bound to purified nAmb a 1 and to rAmb a 1, though 5/11 sera showed significantly higher binding to nAmb a 1 than to rAmb a 1 measuring the optical density at 405 nm (Fig. 3c).

IgE responses to nAmb a 1 and rAmb a 1 were quantified by chimeric ELISA using sera from 89 ragweed-allergic patients from Italy, Austria, Canada and the United States. The chimeric ELISA allowed the absolute levels of IgE to each allergen to be compared using a chimeric mouse human IgE anti-Der p 2 antibody (2B12-IgE) [25]. Overall, there was a significant correlation between IgE antibodies to nAmb a 1 and rAmb a 1 in all groups of patients ($r = 0.790$, $P < 0.001$, $n = 89$, Fig. 3). Approximately 60% of patients showed equivalent IgE ab binding to the natural and the recombinant allergen. A subset of ~40% of patients showed two- to 10-fold greater reactivity to nAmb a 1 than to rAmb a 1. This group included 20/89 patients (22%) with IgE to nAmb a 1 (1–10 IU/mL), but no detectable IgE to rAmb a 1

(< 0.35 IU/mL) (Fig. 4). There did not appear to be any patterns of IgE reactivity that could be distinguished using sera from the four different countries. Thus IgE ab levels in sera from Italy and Austria showed similar binding to nAmb a 1 and rAmb a 1 as those from the United States and Canada.

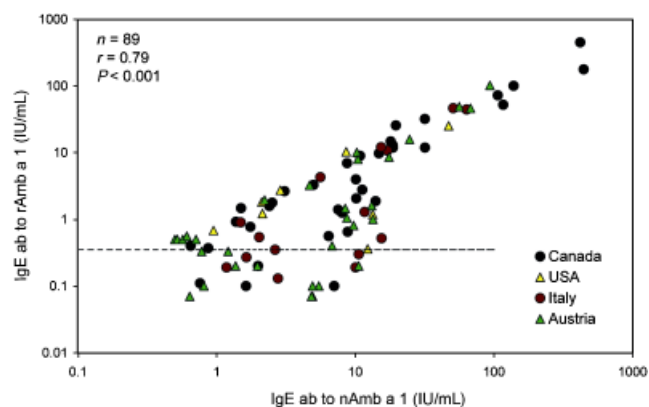
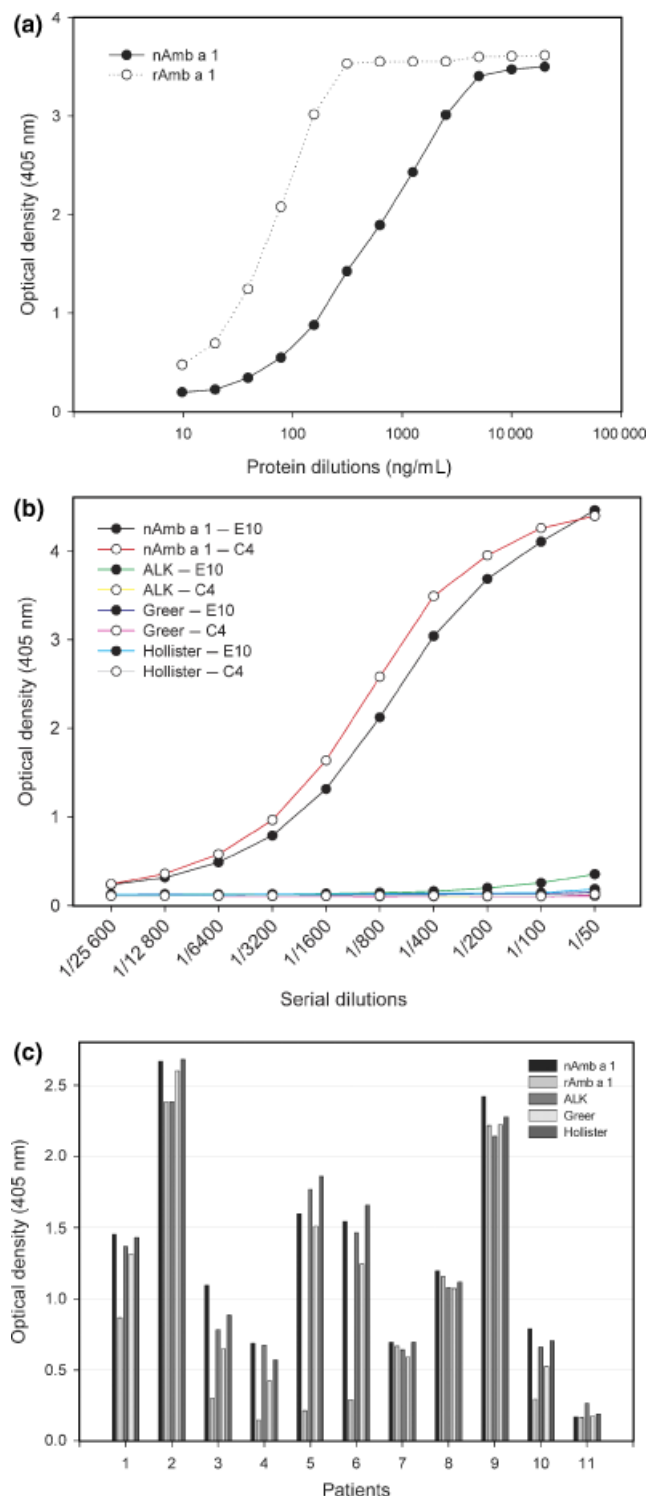


Fig. 4. Comparison of human IgE binding to natural and recombinant Amb a 1. A significant correlation was found between levels of IgE antibodies to natural and recombinant Amb a 1, quantified by chimeric ELISA. Different patient groups were pointed out in various colours. Undetectable levels of specific IgE by chimeric ELISA were assigned a value of <0.35 IU/mL and are indicated with a dashed line.

Discussion

Given the allergenic importance of Amb a 1, it is surprising that there are only two previous reports, 20 years back, of murine mAb raised against either purified nAmb a 1 or against denatured nAmb a 1 [26, 27]. These mAb reacted with epitopes, which were conformation dependent, and the mAb were reported to inhibit human IgE binding to Amb a 1 by up to 80% [26]. Those mAb were not preserved and are no longer available for study. In the present study, we produced mAb against nAmb a 1 and against the *E. coli* expressed rAmb a 1 and compared human IgE ab binding to the natural and recombinant allergen by chimeric ELISA. The panel of mAb reacted strongly on immunoblots with Amb a 1 in ragweed pollen extract, as well as with purified natural and recombinant protein. Three of the mAb reacted exclusively with intact Amb a 1 at 38 kDa, but the majority reacted with the 26 kDa proteolytic product of the molecule as well. Furthermore, it is worth mentioning that the mAb C4 seemed to recognize the intact Amb a 1 protein less efficiently than the 26 kDa band, which may be due to the possibility that the C4 binds to an epitope that is partially buried in the

Fig. 3. Monoclonal antibody ELISA for Amb a 1 detection. (a) Significant difference in the rise of the binding curves for nAmb a 1 and rAmb a 1 were observed using Amb a 1-specific mAb as capture (E10) and detection antibodies (C4). (b) Serial twofold dilutions from natural and recombinant Amb a 1 (0.01–5 µg/mL) and the commercial pollen extracts (1 : 50–1 : 25 600) were used in a two-site mAb ELISA to detect Amb a 1. Detection of Amb a 1 in commercial pollen extracts was not possible using this assay. Monoclonal antibody clones C4 and E10 acted as capture antibody, biotinylated mAb C4 was used for detection. (c) Comparison of specific IgE levels to natural Amb a 1, recombinant Amb a 1 and commercial pollen extracts from ALK, Greer and Hollister-Stier using 11 sera from ragweed-allergic patients. mAb, monoclonal antibodies; nAmb a 1, natural Amb a 1; rAmb a 1, recombinant Amb a 1.

folded full-length protein. Several different combinations of mAb were tested for use in two-site ELISA and mAb E10 (38 kDa reactive) and C4 (38 and 26 kDa) were selected because they showed strong reactivity (Fig. 3a). It was also possible to use mAb C4 as both capture and detector mAb in ELISA, suggesting that this epitope is repeated on Amb a 1 (data not shown). Surprisingly, although these reacted with purified nAmb a 1 and rAmb a 1, the ELISA could not measure Amb a 1 in natural pollen extracts. Various concentrations of ragweed pollen extract and different mAb combinations have been used but still the sandwich ELISA did not work out. The most likely explanation for this is that the mAb were raised against rAmb a 1 and the ELISA had relatively low sensitivity for the natural allergen (~20 µg/mL, Fig. 3a). The mAb clone B7, only directed against the natural protein, could not be used to set up the assay on the first place, because no comparison with rAmb a 1 could be performed. Furthermore, the antibody titre in the cell culture supernatants was not high enough to make an antibody purification lucrative. The results of both the immunoblot and the ELISA experiments demonstrated that the mAb reacted with multiple, non-overlapping epitopes on the Amb a 1 molecule.

Quantitative comparisons of IgE responses to nAmb a 1 and rAmb a 1 were made using the chimeric ELISA. This assay has previously been used to make absolute measurements of IgE ab to mite Groups 1 and 2 allergens and to Fel d 1 [24, 25]. The assay uses a combination of allergen-specific mAb for allergen capture and human IgE ab for allergen detection. In principle, any high-affinity IgG mAb can be used for allergen capture (provided that it does not inhibit IgE binding). The assay is quantified using a control curve of anti-Der p 2 mAb as capture antibody and a chimeric mouse/human IgE anti-Der p 2 antibody for detection, which provides levels of allergen-specific IgE. Using this approach, it was possible to accurately quantify levels of specific IgE to Amb a 1 in IU/mL in a single assay. Because the sera were selected based on their reactivity with nAmb a 1 in ragweed pollen extract, all of them had IgE to purified nAmb a 1. While the chimeric ELISA results showed a highly significant correlation between IgE ab binding to nAmb a 1 and rAmb a 1 ($r=0.79$, $P<0.001$), a significant number of patients (~40%) showed stronger IgE ab binding to the natural allergen than to the recombinant. These data suggest that the rAmb a 1 produced in *E. coli* may not be properly folded and lacks some of the antigenic determinants of the natural protein. Differences between IgE binding to nAmb a 1 and rAmb a 1 did not appear to be explained by geographic differences in sensitization. Ragweed is not that common in Austria and, in keeping with this, many Austrian patients had lower specific IgE to nAmb a 1, and 35% (11/31) did not react with rAmb a 1. The figures for Canadian and Italian patients who did not react with rAmb a 1 were 11% (4/35) and 33% (5/15), respectively.

Previous semi-quantitative studies showed that rAmb a 1 and Amb a 2 produced in *E. coli* under denaturing conditions differed in their ability to bind human IgE, whereas the natural proteins showed similar IgE-binding capacities [17]. The differences in IgE binding to rAmb a 1 would not appear to be explained by isoform differences, because earlier immunoblotting data showed that most patients' IgE reacted with Amb a 1.1 as well as Amb a 1.2 and the Amb a 1.3 isoform (used in the present study). A comparison of the reactivity of T-cells derived from 12 allergic patients to these rAmb a 1 isoforms also suggested that these allergens are all capable of stimulating T-cell proliferation *in vitro* [28].

Our results have significant implications for ragweed allergen standardization and for the development of new diagnostics and vaccines for ragweed allergy. The development of more sensitive, quantitative ELISA for Amb a 1 will require the production of mAb directed against nAmb a 1 to produce mAb that could potentially be used in combination with those reported here. Alternatively, it may be possible to use monoclonal and polyclonal antibodies in combination. This would enable Amb a 1 to be standardized in studies that would parallel those used for allergen standardization in the European Union CREATE project (F. Ferreira, et al., unpublished data). The data presented in the present paper suggest that improvements in the expression of Amb a 1 in *E. coli* need to be made in order to obtain a fully immuno-reactive allergen, which could be used for diagnostic and therapeutic purposes. The limitations of the *E. coli*-expressed protein may be overcome by using another expression system, e.g. *Pichia pastoris* or plant expression systems. Recent clinical trials have shown that purified recombinant grass pollen allergen vaccines can be successfully used to reduce symptom scores and medication use in patients with grass pollen hayfever. Similar trials in ragweed hayfever will be facilitated by further expression of rAmb a 1 and its evaluation using mAb and chimeric ELISA as shown in this study.

Acknowledgements

We are grateful to Dr T. P. King for the generous gift of purified nAmb a 1. This work was supported by the grant S8802 from the Austrian Research Council (F. W. F.).

References

- 1 Boulet LP, Turcotte H, Laprise C *et al.* Comparative degree and type of sensitization to common indoor and outdoor allergens in subjects with allergic rhinitis and/or asthma. *Clin Exp Allergy* 1997; 27:52–9.
- 2 D'Amato G, Spiekma FT, Liccardi G *et al.* Pollen-related allergy in Europe. *Allergy* 1998; 53:567–78.

- 3 Jäger S. Ragweed (*Ambrosia*) sensitisation rates correlate with the amount of inhaled airborne pollen. A 14-year study in Vienna, Austria. *Aerobiologia* 2000; **16**:149–53.
- 4 Singer BD, Ziska LH, Frenz DA, Gebhard DE, Straka JG. Increasing Amb a 1 content in common ragweed (*Ambrosia artemisiifolia*) pollen as a function of rising atmospheric CO₂ concentration. *Funct Plant Biol* 2005; **32**:667–70.
- 5 Stinson KA, Bazzaz FA. CO₂ enrichment reduces reproductive dominance in competing stands of *Ambrosia artemisiifolia* (common ragweed). *Oecologia* 2006; **147**:155–63.
- 6 King TP, Norman PS, Lichtenstein LM. Isolation and characterization of allergens from ragweed pollen IV. *Biochemistry* 1967; **6**:1992–2000.
- 7 Rafnar T, Griffith IJ, Kuo MC, Bond JF, Rogers BL, Klapper DG. Cloning of Amb a I (antigen E), the major allergen family of short ragweed pollen. *J Biol Chem* 1991; **266**:1229–36.
- 8 Rogers BL, Pollock J, Klapper DG, Griffith IJ. Sequence of the proteinase-inhibitor cystatin homologue from the pollen of *Ambrosia artemisiifolia* (short ragweed). *Gene* 1993; **133**: 219–21.
- 9 King TP, Norman PS, Tao N. Chemical modifications of the major allergen of ragweed pollen, antigen E. *Immunochemistry* 1974; **11**:83–92.
- 10 Gleich GJ, Jacob GL. Immunoglobulin E antibodies to pollen allergens account for high percentages of total immunoglobulin E protein. *Science* 1975; **190**:1106–8.
- 11 King TP, Alagon A, Kochoumian L, Kuan J, Sobotka A, Lichtenstein LM. Limited proteolysis of antigens E and K from ragweed pollen. *Arch Biochem Biophys* 1981; **212**:127–35.
- 12 Norman PS, Winkenwerder WL, Lichtenstein LM. Immunotherapy of hay fever with ragweed antigen E: comparisons with whole pollen extract and placebos. *J Allergy* 1968; **42**:93–108.
- 13 Lichtenstein LM, Norman PS, Winkenwerder WL, Osler AG. In vitro studies of human ragweed allergy: changes in cellular and humoral activity associated with specific desensitization. *J Clin Invest* 1966; **45**:1126–36.
- 14 Lichtenstein LM, Norman PS, Winkenwerder WL. A single year of immunotherapy for ragweed hay fever. Immunologic and clinical studies. *Ann Intern Med* 1971; **75**:663–71.
- 15 Lichtenstein LM, Norman PS, Winkenwerder WL. Antibody response following immunotherapy in ragweed hay fever: all-pyrul vs. whole ragweed extract. *J Allergy* 1968; **41**:49–57.
- 16 Lichtenstein LM, Ishizaka K, Norman PS, Sobotka AK, Hill BM. IgE antibody measurements in ragweed hay fever. Relationship to clinical severity and the results of immunotherapy. *J Clin Invest* 1973; **52**:472–82.
- 17 Kuo MC, Zhu XJ, Koury R *et al*. Purification and immunochemical characterization of recombinant and native ragweed allergen Amb a II. *Mol Immunol* 1993; **30**:1077–87.
- 18 Jutel M, Jaeger L, Suck R, Meyer H, Fiebig H, Cromwell O. Allergen-specific immunotherapy with recombinant grass pollen allergens. *J Allergy Clin Immunol* 2005; **116**:608–13.
- 19 Niederberger V, Horak F, Vrtala S *et al*. Vaccination with genetically engineered allergens prevents progression of allergic disease. *Proc Natl Acad Sci USA* 2004; **101** (Suppl. 2):14677–82.
- 20 King TP, Norman PS, Connell JT. Isolation and characterization of allergens from ragweed pollen. II. *Biochemistry* 1964; **3**: 458–68.
- 21 Koehler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 1975; **256**:495–7.
- 22 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**:680–5.
- 23 Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979; **76**:4350–4.
- 24 Ichikawa K, Iwasaki E, Baba M, Chapman MD. High prevalence of sensitization to cat allergen among Japanese children with asthma, living without cats. *Clin Exp Allergy* 1999; **29**:754–61.
- 25 Trombone AP, Tobias KR, Ferriani VP *et al*. Use of a chimeric ELISA to investigate immunoglobulin E antibody responses to Der p 1 and Der p 2 in mite-allergic patients with asthma, wheezing and/or rhinitis. *Clin Exp Allergy* 2002; **32**:1323–8.
- 26 Olson JR, Klapper DG. Two major human allergenic sites on ragweed pollen allergen antigen E identified by using monoclonal antibodies. *J Immunol* 1986; **136**:2109–15.
- 27 Smith JJ, Olson JR, Klapper DG. Monoclonal antibodies to denatured ragweed pollen allergen Amb a I: characterization, specificity for the denatured allergen, and utilization for the isolation of immunogenic peptides of Amb a I. *Mol Immunol* 1988; **25**:355–65.
- 28 Bond JF, Garman RD, Keating KM *et al*. Multiple Amb a I allergens demonstrate specific reactivity with IgE and T cells from ragweed-allergic patients. *J Immunol* 1991; **146**:3380–5.