

Component-resolved *in vitro* diagnosis in carrot allergy: Does the use of recombinant carrot allergens improve the reliability of the diagnostic procedure?

B. K. Ballmer-Weber*, A. Wangorsch†, B. Bohle‡, S. Kaul†, T. Kündig*, K. Fötisch†, R. van Ree§ and S. Vieths†

*Allergy Unit, Department of Dermatology, University Hospital, Zürich, Switzerland, †Division of Allergology, Paul-Ehrlich-Institut, Langen, Germany, ‡Department of Pathophysiology, Medical University of Vienna, Vienna, Austria and §Department of Immunopathology, Sanquin Research, Amsterdam, Netherlands

Summary

Background In Europe, pollen-related food allergy is the most frequent form of food allergy in adults. Reliability of current diagnostic procedures, however, is poor and therapeutic options are not available.

Objectives In the present study, we created a panel of recombinant allergens from carrot and evaluated its potential in component-resolved *in vitro* diagnosis of carrot allergy.

Methods Recombinant (r) Dau c 1.0104, Dau c 1.0201 and Dau c 4 were cloned by a polymerase chain reaction strategy, expressed in *Escherichia coli* and purified. Carrot lipid transfer protein (LTP) was expressed in the yeast *Pichia pastoris*. Sera from 40 carrot-allergic patients were investigated. Twenty-one birch pollen-allergic subjects with negative open provocation to carrot and 20 non-allergic subjects were included as controls. IgE binding to recombinant allergens as well as to cross-reactive carbohydrate determinants (CCD) was measured by ELISA. Cross-reactivity between Dau c 1 isoforms and Bet v 1 was assayed by ELISA inhibition.

Biological activity of the recombinant carrot allergens was assessed by histamine release assay and peripheral blood mononuclear cells stimulation.

Results Ninety-eight percent of the carrot-allergic patients were positive to at least one recombinant allergen; 98% reacted to rDau c 1.0104, 65% to rDau c 1.0201, 38% to rDau c 4 and 20% had IgE against CCD. Specificity using the recombinant allergens was high when compared with non-allergic controls, but low compared with birch-sensitized subjects without carrot allergy. Sensitization to Dau c 1.0201, however, proved to be highly specific for clinically relevant sensitization. Inhibition assays indicated the absence of LTP in carrot root extract, and epitope diversity between Dau c 1.0104, Dau c 1.0201 and Bet v 1.

Conclusions Our panel of recombinant allergens from carrot can provide a standardized tool for *in vitro* diagnosis of carrot allergy, and for epitope studies.

Keywords carrot allergy, component-resolved diagnosis, Dau c 1.0104, Dau c 1.0201, Dau c 4, immunotherapy, LTP, pollen-related food allergy

Submitted 14 October 2004; revised 9 December 2004; accepted 15 February 2005

Introduction

In Europe, pollen-related food allergy is the most frequent form of food allergy in adults and its prevalence has increased over the last few years. Members of the Apiaceae family such as carrots and celery are important elicitors of allergic symptoms [1–4]. Accordingly, 25% of food allergic subjects in Central Europe suffer from carrot allergy [5].

The immunological basis of pollen-related food allergy is IgE cross-reactivity because of the presence of related

proteins sharing a high degree of amino acid sequence identity in pollen (especially birch pollen) and allergenic foods of plant origin such as fruits and vegetables [6]. In our previous studies, we found patients with confirmed carrot allergy, i.e. with a positive double-blind placebo-controlled food challenge, mainly sensitized to the carrot allergen, Dau c 1, which is related to the major birch pollen allergen, Bet v 1, to carrot profilin, Dau c 4, and to cross-reactive carbohydrate determinants (CCD) [4]. So far, three so-called 'isoforms' of the major carrot allergen Dau c 1, i.e. Dau c 1.1, Dau c 1.2 and Dau c 1.3 have been identified, which should be designated, however, variants of Dau c 1 because of their high amino acid sequence identity [2]. Furthermore, a non-birch pollen related protein belonging to the family of non-specific lipid transfer proteins (LTP) has been reported to be

Correspondence: B. K. Ballmer-Weber, MD, Allergy Unit, Department of Dermatology, University Hospital Zurich, Gloriastr. 31, CH-8091 Zurich, Switzerland.

E-mail: barbara.ballmer@usz.ch

an important IgE-binding component in food allergic patients from Italy [7, 8].

Our previous studies indicated that diagnostic procedures in pollen-related food allergy are strongly affected by low sensitivity and specificity of *in vivo* and *in vitro* determination of specific IgE to extracts from plant-derived food [3, 4, 9, 10]. For example, only 20% of true carrot allergic patients were skin prick test positive to a commercial carrot extract, and *in vitro* determination of specific IgE to carrot by CAP-FEIA resulted in a higher sensitivity but was accompanied by a low specificity [4].

In order to offer an efficient treatment to patients with food allergy, the development of a specific immunotherapy (SIT) with food allergens will be required. The low quality of extracts derived from plant food, however, does not allow their use for SIT [11–13]. The use of single allergens or mixtures of recombinant food allergens instead of non-standardized extracts may improve the diagnosis and therapy of food allergy. These molecules are more stable and can be applied in component resolved diagnosis, or specifically and in defined quantities for therapy [9].

In the present study, we have therefore produced the most relevant carrot allergens as recombinant molecules. We have assessed their biological activity, analysed their relevance in a group of carrot-allergic patients, and evaluated the degree of IgE cross-reactivity in pollen allergic patients without carrot allergy. Furthermore, two distantly related isoforms of the major allergen in carrot, Dau c 1, were cloned to assess the potential impact of the use of isoforms on the sensitivity and specificity of *in vitro* diagnosis.

Materials and Methods

Subjects

Eighty-one subjects were included in the study. Sera from 40 carrot allergic patients were selected. Carrot allergy had been confirmed by double-blind placebo-controlled food challenges in 20 patients [4], and the other 20 patients were selected according to a clinical history of carrot allergy and elevated carrot-specific serum IgE determined by CAP-FEIA (Pharmacia Diagnostics, Uppsala, Sweden). Furthermore, sera from 21 birch pollen allergic patients without carrot allergy and 20 healthy controls, both with a negative open provocation with carrots, were analysed. All study participants provided written informed consent.

Open provocation

An open challenge was performed in all birch pollen allergic patients without a history of carrot allergy and all healthy control subjects by ingesting 35 g of raw carrots.

Extracts, recombinant allergens, N-glycans

Recombinant (r) Bet v 1 was purchased from BIOMAY (Vienna, Austria) and birch pollen extract from MAST Diagnostica (Reinfeld, Germany). Carrot extract was prepared from fresh carrot roots as described elsewhere at different pHs (7.4 and 4.0) [4]. For the search of carrot LTP, additional extracts from carrot peel and carrot leaves

were prepared. Milk extract was prepared as described elsewhere [4, 14].

N-linked Glycopeptides with the glycan structure Man α 1-6(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc (MUXF³) and just two to four amino acid residues were prepared by F. Altmann (Vienna, Austria), from extensively digested pineapple stem bromelain, as previously described [15]. The purity of the glycopeptides was checked by matrix-assisted laser desorption ionization time-of-flight mass spectrometry, amino acids and monosaccharides were analysed, and the absence of undigested proteins was confirmed by SDS-PAGE [16–19]. A glycopeptide with the common pentasaccharide core Man α 1-6(Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc (MM), prepared from bovine fibrin, was used as a control [19]. LTP-specific complementary DNA (cDNA) was transferred from a pBluescript vector containing cDNA encoding carrot LTP derived from embryogenic cell cultures of carrot to the *Pichia pastoris* expression vector pPICZ α as described elsewhere [7, 20].

Electrophoresis and immunoblotting

The different carrot extracts were separated by Tricine SDS-PAGE through use of a Mini-Protean cell (Bio-Rad, Munich, Germany) as described [4, 21]. Tank blotting and IgE antibody detection were performed as described [4]. Nitrocellulose strips were incubated overnight with 80 μ L of patients' sera with known IgE reactivity to cherry and hazelnut LTP (serum No. PEI 127, PEI 131) and a control serum from a non-allergic donor in 520 μ L TBS 0.05% Tween20 containing 0.1% bovine serum albumin (BSA). Additional strips were incubated with rabbit anti-carrot LTP (1 : 500), and immunodetection was performed with goat anti-rabbit biotin (1 : 6000) followed by streptavidin phosphatase (Caltag, Burlingame, CA) [7].

Generation of recombinant carrot allergens Dau c 1.0104, Dau c 1.0201 and Dau c 4

First-strand cDNA was synthesized from approximately 1 μ g of total RNA extracted from fresh carrot by using the degenerated NotI-d(T)₁₈ bisfunctional primer provided with the First-Strand cDNA synthesis Kit used for cDNA synthesis (Amersham Biosciences, Freiburg, Germany). For cDNA amplification, the gene-specific primer Dau c 1Nde(+) 5'-GCT CAT ATG GGT GCC CAG AGC CAT TCA CTC GAG ATC-3' (restriction site underlined) was selected for Dau c 1.0104 according to its known cDNA sequence, whereas for Dau c 1.0201 the primer 5'-ATG GGT GTC CAA AAG ACC GTG GTT GAG GCT CCT-3' was designed according to the cDNA sequence of Api g 1.0201 from celeriac [2, 22]. For the profilin Dau c 4 a degenerated oligonucleotide primer Prof5(deg) 5'-ATG TCG TGG CAR RCG TAC GT-3' was used [23]. For the reverse direction, the Not I-d(T)₁₈ primer was applied for Dau c 1.0201 and Dau c 4, and the gene-specific primer Dau c 1Bam(–) 5'-GTC GGA TCC TTA GTT AGC AAT GAG GTA GGC CTC GAT AGC-3' was used for Dau c 1.0104. The first strand cDNA synthesis product was amplified by polymerase chain reaction (PCR) (hot start 94 °C, 2 min, 35 cycles: 30 s denaturation at 94 °C, 1 min annealing at 52 °C, and 1.5 min polymerization at

72 °C, and 5 min final extension at 72 °C). Rapid amplification of cDNA ends (5'RACE) was performed with the Gibco BRL 5'RACE system (Gibco BRL, Eggenstein, Germany) for cloning of Dau c 1.0201 and Dau c 4. First-strand cDNA synthesis was primed with the antisense gene-specific primer 5'-TCT TCG GGG AGC ACA G-3' (Dau c 1.0201) and 5'-GTT CAT CAT AGA CTC C-3' (Dau c 4), respectively. After dC-tailing, cDNA was amplified by PCR using nested second antisense gene-specific primers 5'- GTG TTG TAA ATG GTG TTC TTC AC-3' and 5'-TTC TTA ATG GTC ACA CCT CCA GAG CCC-3', respectively, and the abridged anchor primer provided with the 5'RACE system. The obtained sequences of the two overlapping partial cDNAs were used to generate the terminal primers Dauc102Nde(+) 5'-CGC CAT ATG GGT GTC CAA AAG ACT GAG GTT GAG GC-3' and Dauc102Bam(-) 5'-CC GGA TCC TTA GTT TGC TAG GAG GTA AGC CTC AAC AGC-3' for Dau c 1.0201 and Dauc4Nde(+) 5'-CGC CAT ATG TCG TGG CAA ACA TAC GTT GAC GAT CAC-3' and Dauc4Bam(-) 5'-CC GGA TCC CTA GAG GCC CTG CTC AAT GAG ATA ATC TCC-3' for Dau c 4, respectively, corresponding to the cDNA of the mature proteins. Forward and reverse primers were applied in a PCR with 30.0 µg of cDNA (95 °C for 2 min, 35 cycles of 95 °C for 30 s, 54 °C for 1 min and 72 °C for 1.5 min, followed by a terminal extension at 72 °C for 10 min).

The purified cDNAs were ligated into pCRII-TOPO (Invitrogen, Groningen, the Netherlands) and transformed in *Escherichia coli* Top¹⁰ cells (Invitrogen), and subsequently isolated plasmids were subjected to DNA sequence analysis.

Plasmids were digested with *NdeI* and *BamHI*, for Dau c 1.0104 and Dau c 1.0201 ligated into the pET-16b expression vector (Novagen, Madison, WI, USA), and for Dau c 4 into the pET-30a(+) yielding a deletion of the His-Tag sequence, amplified in NovaBlue Single cells, purified and transformed into *E. coli* BL21(DE3) expression cells (Novagen).

After sequence verification clones were used to produce rDau c 1.0104 and rDau c 1.0201 as N-terminal 10-histidine tagged fusion proteins and rDau c 4 as a non-fusion protein.

The profilin rDau c 4 was purified by means of a poly L-proline column from the cell pellet as described elsewhere [23]. As a deviation from this protocol, profilin solution was dialysed stepwise against decreasing amounts of urea (6, 4, 2 M, no urea) in 1/10 phosphate-buffered saline (PBS). rDau c 1.0104 and rDau c 1.0201 were purified via Ni²⁺-chelate affinity columns (Qiagen, Hilden, Germany).

Amplification of carrot lipid transfer protein DNA

RNA purification and first-strand cDNA synthesis were performed as described above. For cDNA amplification, the gene-specific primers were selected according to the known sequence of carrot LTP(+) 5'-ATGGGAGTTCTAAGATC-CAGCTTTGTAGCC-3' and LTP(-) 5'-TCACACCACC-CTGTTGCAATCGGTGGTGGG-3'. Genomic DNA was produced from carrot powder using the SureFood-PREP PlantX kit (Congen Biotechnologie, Berlin, Germany), and the same primers were applied to amplify genomic DNA encoding for carrot LTP.

Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy of recombinant Dau c 1.0104 and Dau c 1.0201 was performed on a Jasco J-810S spectropolarimeter (Jasco, Groß-Umstadt, Germany) at 20 °C with a stepwidth of 0.2 nm and a bandwidth of 1 nm. The spectral range was 190–260 nm at 50 nm/min. Six scans were accumulated. The protein concentration was 5.5 µM in 10 mM KH₂PO₄, pH 7.0.

In vitro determination of allergen-specific serum immunoglobulin E reactivity and inhibition enzyme-linked immunosorbent assay

CAP Specific IgE to carrot, birch pollen, rBet v 1 and rBet v 2 were measured through use of the CAP FEIA system (Pharmacia Diagnostics).

Standard enzyme-linked immunosorbent assay IgE binding to recombinant carrot allergens was determined by a direct solid-phase ELISA in polystyrene microtitre plates (MaxisorpTM, Nunc, Roskilde, Denmark) coated overnight at 4 °C with 50 ng protein per well in 100 µL PBS, blocked with 200 µL PBS/BSA (1%) for 1 h and incubated with 100 µL of serum (1 : 2 diluted, o/n at room temperature (RT)). Detection of bound-specific IgE was performed with a rabbit anti-human IgE antiserum (DAKO, Copenhagen, Denmark; 100 µL, 1 : 4000, 90 min at RT) and a Biotin-conjugated goat anti-rabbit IgG (DAKO) as secondary antibody (100 µL, 1 : 6000, 1 h at RT). For visualization, Streptavidin-HRP (Calbiochem 189733, Bad Soden, Germany; 100 µL, 1 : 10000, 30 min at RT) was applied using 3,3',5,5'-tetramethylbenzidine (100 µL, 20–30 min at RT in the dark) as substrate. Reaction was stopped with 50 µL 6 N H₂SO₄ and optical density (OD) was measured at 450 nm. The results were regarded as positive if the absorbance obtained was more than twofold of the OD of a non-atopic control subject. Experiments were run in duplicates.

Glycan enzyme-linked immunosorbent assay IgE specific for carbohydrate structures were detected by a Glycan ELISA using glycopeptides MUXF³ and MM covalently linked to microtitre plates (CovalinkTM, Nunc) as described [16].

Enzyme-linked immunosorbent assay inhibition For inhibition ELISA coating and blocking was performed as for standard ELISA. To adjust for an approximately identical amount of specific IgE per test, patients' sera were serially diluted to an OD between 0.800 and 1.2 in the standard ELISA. The following inhibitors were applied: rDau c 1.0104, rDau c 1.0201 and rBet v 1 at concentrations of 250, 100, 10, 1 µg/mL, LTP at concentrations of 66, 10, 1.3 and 0.18 µg/mL, birch pollen extract at concentrations of 1000, 100, 10, 1 µg/mL, and carrot extract at concentrations of 500, 50, 5, 0.5 µg/mL. Fifty microlitres of each serum and 50 µL of the inhibitors were incubated for 1 h at RT. Milk extract was used as inhibition control. Non-specific binding (NSB) was determined with serum from a non-atopic subject. The uninhibited sera (*B*₀), preincubated sera and controls were transferred to the coated plates and residual IgE binding capacity was determined. Percentage of inhibition was

calculated (% inhibition = $[\text{OD}(\text{Bo}) - \text{OD}(\text{Serum with inhibitor})] / [\text{OD}(\text{Bo}) - \text{NSB}] \times 100$).

Basophil histamine release

The histamine release from stripped and passively sensitized basophils was performed as described with several modifications as described by Kleine Budde [24] and Fötisch et al. [25].

Proliferation assays

Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood by Ficoll-Hypaque density gradient centrifugation (Pharmacia Diagnostics). PBMC (2×10^5) were cultured in triplicates in 96-well plates (Nunc, Denmark) in 200 μL of serum-free Ultra Culture Medium (BioWhittaker, Walkersville, MD, USA) supplemented with 2 mM/L-glutamine and 2×10^{-5} M 2-ME for 6 days at 37 °C and 5% CO_2 in a humidified atmosphere. rDau c 1.0104 and rDau c 1.0201 were titrated in the concentration range of 0.07–10 $\mu\text{g}/\text{mL}$, rBet v 1 in the range of 3.18–25 $\mu\text{g}/\text{mL}$, and carrot extract in the range of 1.25–10 $\mu\text{g}/\text{mL}$. During the last 16 h of culture [^3H]-thymidine (0.5 μCi per well) was added and the incorporated radioactivity was measured by scintillation counting. The stimulation index (SI) was calculated as ratio between cpm obtained in cultures with autologous PBMC plus allergen and cpm obtained in cultures containing PBMC.

Data analysis

To compare IgE reactivity of carrot and birch pollen allergic patients to recombinant allergens the Kolmogorov–Smirnov test was used [26].

Results

Patients

A total of 40 carrot allergic patients (mean age: 32 ± 11 years) entered the study. Case histories with respect to carrot allergy contained the following reactions: isolated oral allergy syndrome ($n = 21$), angioedema ($n = 9$), urticaria ($n = 2$), dyspnoea ($n = 2$), thoracic tightness ($n = 3$), dysphagia ($n = 2$), dysphonia ($n = 1$), cough ($n = 1$), rhinitis or conjunctivitis ($n = 3$), gastrointestinal problems ($n = 1$) and vertigo ($n = 1$). Control group 1 consisted of 21 patients with birch pollinosis but no carrot allergy (mean age: 32.3 ± 9.5 years) and control group two of 20 non-atopic subjects (mean age: 32 ± 4 years). In all participants of control groups 1 and 2, the lack of carrot allergy was confirmed by a negative open provocation with carrots.

Production and characterization of recombinant carrot allergens

Applying a PCR strategy, two isoforms of the major carrot allergen Dau c 1, i.e. Dau c 1.0104 and Dau c 1.0201, were cloned. Isoform Dau c 1.0104 (GenBank accession No. Z81362), has previously been described as Dau c 1.3 [2]. It consists of 154 amino acid residues with a deduced molecular

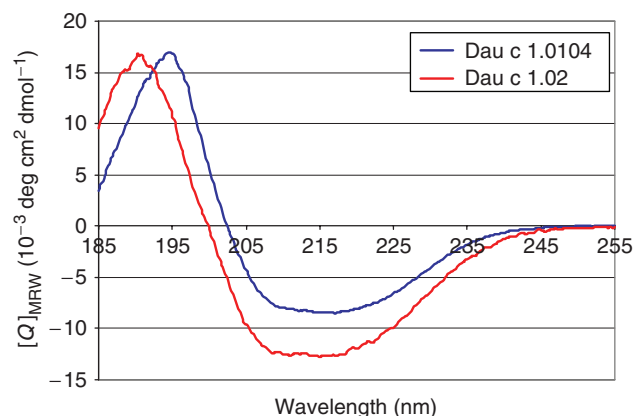


Fig. 1. Circular dichroism (CD) spectra of carrot allergens Dau c 1.0104 and Dau c 1.0201.

mass of 16 093 kDa and has a predicted isoelectric point of 4.39. The amino acid sequence identity is 81% with the major allergen in celery, Api g 1.0101 (accession No. Z75662), but only 37% with Bet v 1a (accession No. X15877) [27].

The cDNA of the isoform Dau c 1.0201 (accession No. AF456481) consisted of 465 bp, coding for a 153 amino acid protein. The calculated molecular weight is 16.4 kDa and the theoretical isoelectric point 4.4. Dau c 1.0201 is 50% identical with Dau c 1.0104, 92.4% with Api g 1.0201 and 38% with Bet v 1a. Sequence identity with other Bet v 1-related food proteins ranges from 38% (Pyr c 1 from pears) to 61% (PR1-3 from parsley).

Moreover, we cloned the carrot profilin that has been shown to bind IgE of 20% of carrot allergic patients [4]. The complete amino acid sequence of the carrot profilin, Dau c 4 (accession No. AF456482), comprises a protein with a calculated molecular weight of 14 425 kDa and an isoelectric point of 4.75. Comparison of the Dau c 4 amino acid sequence with the SWALL Protein Database shows identities ranging from 74% to 91% with many other plant profilins, i.e. of 91% with profilin from celery (Api g 4) and 81% with birch pollen profilin (Bet v 2).

Purified rDau c 1.0104, rDau c 1.0201 and rDau c 4 were demonstrated to be >95% pure as assessed by denaturing SDS-PAGE and Coomassie blue staining (not shown). The secondary structure of the Bet v 1 homologues was analysed by CD spectroscopy (Fig. 1).

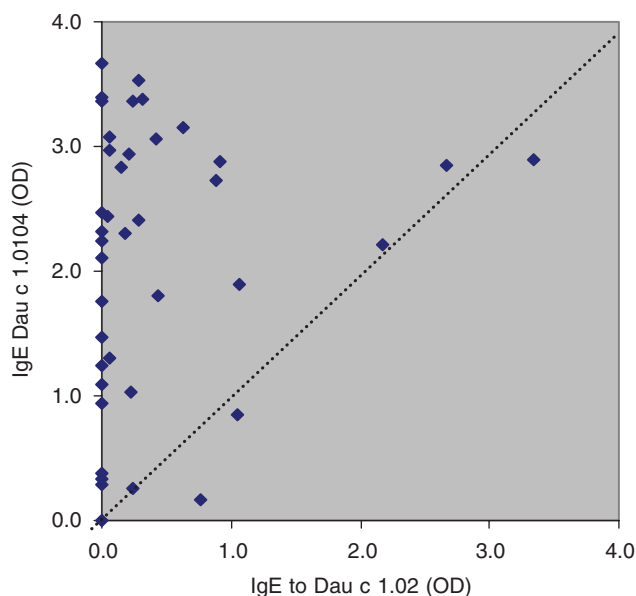
Both recombinant Dau c 1.0104 and Dau c 1.0201 (designated as Dau c 1.02 in all figures) were found to be folded but showed different contents of secondary structure elements.

Component-resolved in vitro diagnosis confirms Dau c 1 isoforms as major carrot allergens

To assess the pattern of IgE reactivity to the recombinant carrot allergens and the N-glycans, sera of carrot allergic patients, pollen allergic patients without carrot allergy and non-allergic subjects were screened by IgE ELISA. The IgE reactivity pattern for each group is summarized in Table 1 together with the results of additional CAP determinations. Ninety-eight percent of carrot allergic patients were IgE positive to rDau c 1.0104 and 65% to rDau c 1.0201. Thus, both isoforms met the criteria for being major allergens. IgE

Table 1. Specific IgE reactivity to carrot allergens and birch pollen allergens determined by CAP and ELISA

	Carrot allergic <i>n</i> (%)	Birch pollen allergic <i>n</i> (%)	Non-atopic <i>n</i> (%)
CAP results			
Carrot	39/40 (98%)	9/21 (43%)	0
Birch pollen	39/40 (98%)	21/21 (100%)	0
rBet v 1	39/40 (98%)	20/21 (95%)	0
rBet v 2	13/39 (33%)	7/21 (33%)	0
ELISA results			
Dau c 1.0104	39/40 (98%)	8/21 (38%)	0
Dau c 1.0201	26/40 (65%)	1/21 (5%)	1/20 (5%)
Dau c 4	15/40 (38%)	5/19 (26%)	0
MUXF ³	8/40 (20%)	2/21 (10%)	1/20 (5%)

**Fig. 2.** Correlation between IgE reactivity to rDau c 1.0104 (optical density, OD) and to rDau c 1.0201 (OD) for each carrot allergic patient as assessed by means of standard ELISA: IgE reactivity to Dau c 1.0104 is significantly stronger than to Dau c 1.0201 ($P < 0.0001$).

reactivity to rDau c 1.0201 ($OD\ 0.417 \pm 0.746$) was significantly lower ($P < 0.0001$) as compared with rDau c 1.0104 ($OD\ 2.084 \pm 1.094$) in 34 patients (Fig. 2). Six sera (15%) showed a similar IgE-binding capacity to both Dau c 1 isoforms. Birch pollen allergic patients without carrot allergy recognized the same components but with clearly lower prevalence (Table 1) and intensity. Mean IgE reactivity of birch pollen allergic patients without carrot allergy to Dau c 1.0104 ($OD\ 0.209 \pm 0.384$) and to rDau c 1.0201 ($OD\ 0.011 \pm 0.048$) was significantly lower than observed for the respective allergens in carrot allergic patients ($P < 0.001$ and $P = 0.0004$, respectively). Just one single birch pollen allergic patient without carrot allergy showed IgE reactivity to rDau c 1.0201. Thus, sensitization to Dau c 1.0201 was found to be much more specific for carrot allergy (specificity of 95%) than sensitization to Dau c 1.0104. None of the non-atopic control subjects reacted with the recombinant carrot allergens Dau c 1.0104 and Dau c 4, one patient was positive for Dau c 1.0201 confirming for those allergens a specificity of the test procedure in regard to non-atopic patients of 100%

and 95%, respectively. Twenty percent of carrot allergics and 10% of the birch allergic controls had IgE against the ubiquitous plant N-glycan MUXF.

Absence of detectable lipid transfer protein in mature carrots

Using sera from 20 subjects with confirmed carrot allergy we were unable to detect in carrot extract an IgE-reactive band corresponding to the expected molecular weight of LTP (9 kDa). To clarify this discrepancy, immunoblotting with extracts of carrot root, peel and leaves extracted at pH 7.4 and pH 4.0 using sera of patients sensitized to cherry and hazelnut LTP was performed and did not result in any IgE reactivity against a protein with the molecular weight in the range of LTPs. A rabbit IgG antibody raised against recombinant LTP did not recognize any protein in either extract (results not shown). IgE reactivity against rLTP derived from embryogenic cell cultures was measured in 28% of carrot allergic patients. The serum with the highest IgE reactivity ($OD\ 3.265$) was further investigated by an ELISA inhibition experiment. No inhibition was observed using carrot extract as inhibitor. A self-inhibition of 91% confirmed the specificity of the reaction. Moreover, we analysed the presence of mRNA coding for carrot LTP by RT-PCR using gene specific primers corresponding to the sequence of carrot LTP (accession No. M64746). No amplicon was obtained. Carrot LTP, however, could be amplified from genomic DNA using the same primers. Accordingly, LTP is not present in edible parts and leaves of adult carrot plants and thus not an allergen for carrot allergic patients.

Cross-reactivity studies indicate the recognition of Bet v 1-unrelated epitopes by a subgroup of patients

To assess IgE cross-reactivity between the two carrot isoforms, rDau c 1.0104 and rDau c 1.0201, and rBet v 1, ELISA inhibition experiments were performed with sera of 24 carrot allergic patients. With rDau c 1.0104 as solid-phase antigen, the overall self-inhibition was $91.7 \pm 4.7\%$ (data not shown). In 19/24 sera, rDau c 1.0201 reduced IgE reactivity to rDau c 1.0104 by just $26.7 \pm 17.4\%$, whereas an inhibition of $75.0 \pm 7.0\%$ was observed with the remaining five sera. In a subset of patients ($n = 5$) IgE binding to rDau c 1.0104 was only partially reduced by pre-incubation with rBet v 1, i.e. $44.4 \pm 8.7\%$ and by birch pollen extract, i.e. $44.7 \pm 11.9\%$, pointing to the existence of Bet v 1 unrelated epitopes on Dau c 1.0104. In the remaining 19 patients, pre-incubation of the sera with rBet v 1 and birch pollen extract inhibited IgE binding to Dau c 1.0104 by $84.8 \pm 8.9\%$ and $86.8 \pm 8.3\%$, respectively. Overall three different inhibition patterns were observed which are summarized in Fig. 3: (A) Inhibition of IgE binding to Dau c 1.0104 of $>60\%$ by Dau c 1.0201 and Bet v 1a or birch pollen extract, respectively, in four patients indicating recognition of similar epitopes by IgE of those patients on the respective allergens (Fig. 3a) and (B) inhibition of IgE binding to Dau c 1.0104 by Bet v 1a and birch pollen extract of $>60\%$ but by Dau c 1.0201 of $<60\%$ (Fig. 3b) in 16 patients and finally (C) low inhibition of IgE binding to Dau c 1.0104 of $<60\%$ by Bet v 1a, birch pollen extract and Dau c 1.0201 in four patients (Fig. 3c). A

representative inhibition experiment for each of the three patterns is depicted in Fig. 3.

To elucidate the specificity of IgE binding to Dau c 1.0201 in carrot allergic patients we measured cross-reaction between

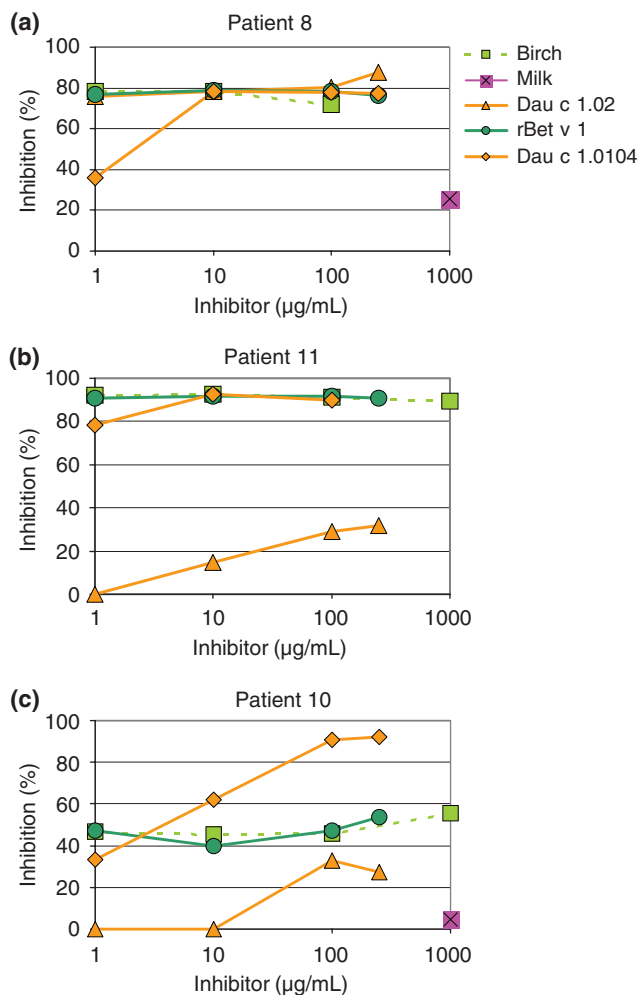


Fig. 3. Inhibition of IgE binding to Dau c 1.0104 by ELISA. Three distinct inhibition patterns reflect the diversity of the IgE response. (a) Inhibition of IgE binding to Dau c 1.0104 of >60% by Dau c 1.0201 and Bet v 1a or birch pollen extract, respectively, indicating recognition of similar epitopes on the respective allergens ($n = 4$); (b) inhibition of IgE binding to Dau c 1.0104 by Bet v 1a and birch pollen extract of >60% but by Dau c 1.0201 of <60% ($n = 16$), (c) low inhibition of IgE binding to Dau c 1.0104 of <60% by Bet v 1a, birch pollen extract and Dau c 1.0201 ($n = 4$).

Dau c 1.0201 and Bet v 1 with immobilized Dau c 1.0201 using sera of five patients. Self-inhibition with rDau c 1.0201 was $81.7 \pm 7.4\%$. In 4/5 patients IgE binding to Dau c 1.0201 was not or weakly inhibited by Dau c 1.0104 ($11.0 \pm 14.1\%$) and was lowered by 71.9% in the fifth patient. In three of five patients there was no or only a weak inhibition of IgE binding to Dau c 1.0201 by rBet v 1 ($6.6 \pm 9.3\%$) or birch pollen extract ($16.9 \pm 13.0\%$).

Recombinant carrot allergens show biologic activity in histamine release experiments

Histamine release experiments with stripped basophils were performed with sera of seven patients. Table 2 shows the maximal release (%) for each allergen tested in relation to total histamine release and the allergen concentration (ng/mL) inducing 50% of maximal release. All recombinant allergens tested were able to induce mediator release and were thus biologically active. All sera showing specific release also contained IgE specific for the respective allergen as indicated by the results of the IgE ELISA.

Both Dau c 1 isoforms share similar peripheral blood mononuclear cells stimulation capacity

Freshly isolated PBMC from 10 allergic individuals were stimulated with different concentrations of rDau c 1.0104, rDau c 1.0201, rBet v 1 and carrot extract, respectively. Comparing the SI at different concentrations of the carrot allergens revealed no difference between both isoforms (Fig. 4). The maximum stimulatory concentrations of rBet v 1 ($12.5 \mu\text{g/mL}$) and carrot extract ($5 \mu\text{g/mL}$) are shown as controls (Fig. 4).

Discussion

To identify the major allergens in an allergenic source is an essential prerequisite for the production of diagnostic or therapeutic allergen-derived material. Therefore, the first aim of our study was to analyse the sensitization pattern of carrot allergic patients.

We have now cloned, expressed and characterized the carrot allergens identified in our former study, i.e. the Bet v 1 related allergen Dau c 1 (isoform Dau c 1.0104) and the carrot profilin, Dau c 4. To assess IgE reactivity to cross-

Table 2. Histamine release assay with stripped basophils and sera of carrot allergic patients

Patient number	16		14		7		6		19		37		5	
	Max (%)	1/2 Max (ng/mL)	Max (%)	1/2 Max (ng/mL)	Max (%)	1/2 Max (ng/mL)	Max (%)	1/2 Max (ng/mL)	Max (%)	1/2 Max (ng/mL)	Max (%)	1/2 Max (ng/mL)	Max (%)	1/2 Max (ng/mL)
Birch pollen extract	68.7	n.p.	48.3	n.p.	58	n.p.	68.2	n.p.	83.6	n.p.	62	n.p.	n.d.	—
Carrot extract	61.5	2000	39.3	500	57.3	<1000	17.8	3000	71.2	30	18.7	6000	11.5	8000
Dau c 1.0104	69.1	0.7	19.7	1000	<10	n.p.	<10	n.p.	54.6	0.3	<10	n.p.	14.2	80
Dau c 1.0201	33.2	0.5	<10	2000	<10	n.p.	12.5	n.p.	65.6	<0.1	<10	n.p.	<10	3
Dau c 4	<10	30	22.7	n.p.	42.9	n.p.	15.1	n.p.	11.2	10	16.5	n.p.	<10	n.p.

Results are given as ratio (%) of maximal allergen induced release per total histamine release and as concentration (ng/mL) inducing 50% of maximal release. n.p.: estimation of 1/2 maximal release not possible, either because decreasing segment of the release curve was not reached by highest dilution (e.g. Bet v 1), or because of a weak maximal release.

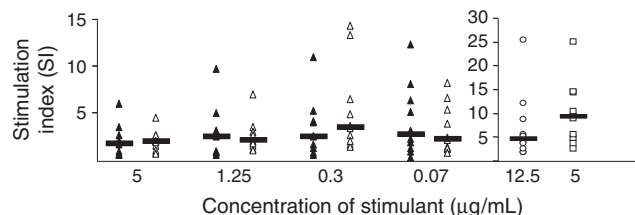


Fig. 4. Proliferative responses of peripheral blood mononuclear cells (PBMC) from 10 allergic individuals to titrated concentrations of rDau c 1.0104 (Δ) and rDau c 1.0201 (▲). Both isoforms induced comparable SI. Maximum proliferative responses to rBet v 1 (○) and carrot extract (□) are shown as controls.

reactive carbohydrate determinants we used N-linked glycopeptides purified from pineapple stem bromelain. IgE reactivity to that panel of allergens was assessed in 40 carrot allergic patients with a positive DBPCFC to carrots or a clearly positive case history. Dau c 1.0104 was confirmed to be the major allergen since 98% of carrot allergic patients were sensitized to that protein [2, 4]. Thirty-eight percent of the patients recognized Dau c 4 and 20% displayed IgE specific for CCD. Despite a lower prevalence of CCD sensitization that was probably caused by the use of a purified CCD antigen in the present study, the results were in good accordance with our previous data [4].

A recombinant carrot LTP was used as a reference compound in recent studies on allergenic food LTPs, and LTP was reported to be a food allergen in carrot allergic patients from Mediterranean countries [7, 8]. Our experiments, however, demonstrated that LTP is not expressed at detectable levels in the edible parts of carrots and is therefore unlikely to play a role as a food allergen. Repeated extractions from carrot roots and other parts of the plant revealed the absence of LTP which was in accordance with RT-PCR data, while PCR with genomic DNA confirmed the presence of the LTP gene in carrots. Asero et al. [7] reported IgE binding to recombinant carrot LTP in 21/34 sera from patients with multiple food allergies, but negative case histories of carrot allergy. This observation most likely results from clinically insignificant cross-reaction of IgE raised for instance against peach LTP, Pru av 3. The absence of carrot LTP in mature carrots most likely explains the negative case histories in those patients. Our findings suggest that measurement of IgE antibodies against carrot LTP has no relevance on clinical routine diagnostic approaches. We found (mostly weak) IgE binding to recombinant carrot LTP in 28% of carrot allergic patients. Despite a positive self-inhibition, we found no inhibition with the cherry LTP rPru av 3 derived from *E. coli* (results not shown). One may speculate that false-positive IgE binding to the yeast expressed carrot LTP may have been caused by yeast-specific glycans that are not present on natural LTPs in plants.

Furthermore, we have cloned, sequenced and characterized a second isoform of Dau c 1, Dau c 1.0201, which was recognized by IgE of 65% of carrot allergic patients but depicted a statistically significant lower IgE binding capacity than Dau c 1.0104 (Fig. 2).

Sensitivity of using the recombinant carrot allergens for an ELISA assay was 98%. All 98% of carrot allergic patients were positive for Dau c 1.0104. Hence, testing for a second isoform of the major carrot allergen or profilin did not

improve sensitivity of the *in vitro* assay. Only one of the carrot allergic patients was IgE negative for Dau c 1.0104 and that patient was specifically sensitized to cross-reactive carbohydrate determinants. No correlation was observed between sensitization profile, levels of specific IgE, and presence or severity of symptoms (results not shown).

To investigate the extent of clinically insignificant cross-reactions 21 birch pollen allergic patients without carrot allergy were included. Those patients recognized the same allergens as carrot allergic patients but with a much lower frequency. However, on the level of the individual patient, the specificity of serological diagnosis is still affected by this clinically insignificant cross-reactivity. Most interestingly, Dau c 1.0201 was recognized by IgE of only one single birch pollen allergic patient without carrot allergy, resulting in a specificity of 95% in relation to birch pollen allergic patients without carrot allergy.

Consequently, our data suggest that certain isoforms of pollen-related food allergens may be more closely related to clinical relevant sensitization.

It is a generally accepted concept that in pollen-related food allergy primary sensitization is initiated by pollen allergens, and plant food allergy is induced secondarily because of the occurrence of related proteins in plant food. IgE-binding epitopes on pollen and plant food allergens are thought to be very similar. Indeed, our inhibition studies showed that IgE-binding to homologous carrot allergens was inhibited by preincubating the sera of carrot allergic patients with rBet v 1a. However, as observed in our earlier study, this was not true for all patients [4]. In a subset of patients binding to the carrot allergens was only partially inhibited by Bet v 1 or birch pollen extract. Also, Dau c 1.0201 proved to be a weak inhibitor of IgE-binding to Dau c 1.0104 and vice versa in a major subgroup of carrot allergic patients. Our results indicate that on Bet v 1 and each Bet v 1 related carrot allergen a different and individual set of IgE epitopes is present and that recognition of IgE-binding epitopes is highly patient-specific. However, as none of our patients had IgE to Dau c 1 without being sensitized to Bet v 1, our data do not clearly point towards a primary sensitizing capacity of the food allergens.

Hayfever may be efficiently treated in 80–90% of patients by allergen SIT with pollen extracts [28, 29]. Despite high structural similarity of birch pollen and food allergens, SIT with pollen extract in our hands rarely has a significant effect on decrease in severity of food allergy, in particular to carrot and celery (clinical observations in the Allergy Unit Zürich). Literature data, however, are contradictory. Most studies published so far have been performed in apple allergic patients with birch sensitization. In some studies a reduction of food-induced symptoms was observed in patients under SIT with birch pollen extract [30–32]. Others could not find a significant effect of pollen SIT on the course of associated food allergy or even observed a sensitization to plant foods under SIT, and in just one of these studies the severity of adverse food reactions was assessed under well controlled conditions, i.e. DBPCFC [32, 33].

Taken together these results are in accordance with our findings on the B cell level that at least a subgroup of pollen allergic patients with food allergy may be sensitized to isoforms or epitopes on food allergens that are distantly

related or not cross-reactive to pollen allergens. This effect may be more pronounced for pollen-related food allergens with a low degree of sequence identity to Bet v 1 such as Dau c 1 from carrot or Api g 1 from celery. Our results demonstrate that both Dau c 1 isoforms, i.e. the high IgE binding Dau c 1.0104 and the lower IgE binding Dau c 1.0201, were able to stimulate T cells to a similar extent. So far, just two Bet v 1 homologous food allergens have been shown to induce T cell stimulation, i.e. Mal d 1 from apple and Api g 1 from celery [34, 35]. Most importantly, Dau c 1.0201 was identified as an allergen with reduced IgE binding but sustained T cell stimulatory capacity and may thus represent a tool for SIT of carrot allergy.

In summary, our data suggest firstly, that component-resolved *in vitro* diagnosis based on recombinant allergen molecules and their isoforms harbours a great potential to improve both specificity and sensitivity of food allergy diagnosis. Secondly, the T cell stimulatory capacity of pollen-related carrot allergens detected in this study may predispose them as candidate vaccines for patients in whom pollen-related food allergy is inefficiently treated by birch pollen immunotherapy.

Acknowledgements

We are grateful for the excellent technical assistance by Astrid Radakovics, Irene Cuhat, Marie-Claire Weber and Susan Marti, for providing purified N-glycans to Friedrich Altmann, Universität für Bodenkultur, Vienna, Austria, and for help in statistical analysis by Dr P. Volkers, Paul-Ehrlich-Institut, Germany.

B.K. Ballmer-Weber was a recipient of a grant for the promotion of academic young people of the University Zürich (Stiefel-Zangger-Stiftung) and of the Swiss National Foundation (32-66308.01). Furthermore, the study was supported by a the grant SFB F018-07 from the Fonds zur Förderung der Wissenschaftlichen Forschung, Vienna, Austria, to Barbara Bohle, and by the Deutsche Forschungsgemeinschaft (VI 165/2-4).

References

- Dreborg S, Foucard T. Allergy to apple, carrot and potato in children with birch-pollen allergy. *Allergy* 1983; 38:167–72.
- Hoffmann-Sommergruber K, O'Riordain G, Ahorn H et al. Molecular characterization of Dau c 1, the Bet v 1 homologous protein from carrot and its cross-reactivity with Bet v 1 and Api g 1. *Clin Exp Allergy* 1999; 29:840–7.
- Ballmer-Weber BK, Vieths S, Lüttkopf D, Heuschmann P, Wüthrich B. Celery allergy confirmed by double-blind, placebo-controlled food challenge: a clinical study in 32 subjects with a history of adverse reactions to celery root. *J Allergy Clin Immunol* 2000; 106:373–8.
- Ballmer-Weber BK, Wüthrich B, Wangorsch A, Fösch K, Altmann F, Vieths S. Carrot allergy: double-blind, placebo-controlled food challenge and identification of allergens. *J Allergy Clin Immunol* 2001; 108:301–7.
- Etesamifar M, Wüthrich B. IgE-vermittelte Nahrungsmittelallergie bei 383 Patienten unter Berücksichtigung des oralen Allergiesyndroms. *Allergologie* 1998; 21:451–7.
- Ebner C, Hirschwehr R, Bauer L et al. Identification of allergens in fruits and vegetables: IgE cross-reactivities with the important birch pollen allergens Bet v 1 and Bet v 2 (birch profilin). *J Allergy Clin Immunol* 1995; 95:962–9.
- Asero R, Mistrello G, Roncarolo D et al. Lipid transfer protein: a pan-allergen in plant derived foods that is highly resistant to pepsin digestion. *Int Arch Allergy Immunol* 2000; 122:20–32.
- Asero R, Mistrello G, Roncarolo D et al. Immunological cross-reactivity between lipid transfer proteins from botanically unrelated plant-derived foods: a clinical study. *Allergy* 2002; 57:900–6.
- Ballmer-Weber BK, Scheurer S, Fritsche P et al. Component resolved diagnosis using recombinant allergens in cherry allergic patients. *J Allergy Clin Immunol* 2002; 110:167–73.
- Mittag D, Vieths S, Vogel L et al. Birch pollen-related allergy to soybean: clinical investigation and molecular characterization of allergens. *J Allergy Clin Immunol* 2004; 113:148–54.
- Van Ree R, Akkedaas JH, van Leeuwen WA et al. New perspectives for the diagnosis of food allergy. *ACI Int* 2000; 12:7–12.
- Rudeschko O, Fahlbusch B, Schlenvoigt G, Hermann D, Vieths S, Jäger L. Investigation of the stability of apple allergen extracts. *Allergy* 1995; 50:575–80.
- Jankiewicz A, Aulepp H, Baltes W et al. Allergic sensitisation to native and heated celery root in pollen-sensitive patients investigated by skin test and IgE binding. *Int Arch Allergy Immunol* 1996; 111:268–78.
- Vieths S, Schöning B, Petersen A. Characterization of the 18 kDa apple allergen by two-dimensional immunoblotting and microsequencing. *Int Arch Allergy Immunol* 1994; 104:399–404.
- Tretter V, Altmann F, März L. Peptide-N⁴-(N-acetyl- β -glucosaminyl) asparagine amidase F cannot release glycans with fucose attached $\alpha 1 \rightarrow 3$ to the asparagine-linked N-acetyl-glucosamine residue. *Eur J Biochem* 1991; 199:647–52.
- Fötisch K, Altmann F, Haustein D, Vieths S. Involvement of carbohydrate epitopes in the IgE response of celery-allergic patients. *Int Arch Allergy Immunol* 1999; 120:30–42.
- Wilson IBH, Altmann F. Structural analysis of N-glycans from allergenic grass, ragweed and tree pollens. Core $\alpha 1,3$ -fucose and xylose present in all pollens examined. *Glycoconjugate J* 1998; 15:1055–70.
- Altmann F. Determination of amino acid sugars and amino acids in glyco-conjugates using pre-column derivatization with o-phthalaldehyde. *Anal Biochem* 1992; 204:215–9.
- Wilson IBH, Harthill JE, Mullin NP, Ashford DA, Altmann F. Core $\alpha 1,3$ -fucose is a key part of the epitope recognized by antibodies reacting against plant N-linked oligosaccharides and is present in a wide variety of plant extracts. *Glycobiology* 1998; 8:651–61.
- Sterk P, Booij H, Schellekens GA, Van Kammen A, De Vries SC. Cell-specific expression of the carrot EP2 lipid transfer protein gene. *Plant Cell* 1991; 3:907–21.
- Schägger Hv, Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 1987; 166:368–79.
- Hoffmann-Sommergruber K, Ferris R, Pec M et al. Characterization of Api g 1.0201, a new member of the Api g 1 family of celery allergens. *Int Arch Allergy Immunol* 2000; 122:115–23.
- Scheurer S, Wangorsch A, Haustein D, Vieths S. Cloning of the minor allergen Api g 4, profilin from celery (*Apium graveolens*) and its cross-reactivity with birch pollen profilin Bet v 2. *Clin Exp Allergy* 2000; 30:962–71.
- Kleine Budde I, de Heer PG, van der Zee JS, Aalberse RC. The stripped basophil histamine release bioassay as a tool for the detection of allergen-specific IgE in serum. *Int Arch Allergy Immunol* 2001; 126:277–85.

- 25 Fötisch K, Westphal S, Lauer I et al. Biological activity of IgE specific for cross-reactive carbohydrate determinants (CCD). *J Allergy Clin Immunol* 2003; 111:889–96.
- 26 Rasch D, Herrendörfer G, Bock J, Victor N, Guiard V. *Verfahrensbibliothek: Versuchsplanung und Auswertung. part I/II.* Munich: Odenburg Verlag, 1998.
- 27 Breiteneder H, Pettenburger K, Bito A et al. The gene coding for the major birch pollen allergen Bet v 1, is highly homologous to pea disease resistance response gene. *EMBO J* 1989; 8:1935–8.
- 28 Bousquet J, Becker WM, Hejjaoui A et al. Differences in clinical and immunological reactivity of patients allergic to grass pollen and to multiple pollen species. Efficacy of a double blind placebo controlled specific immunotherapy with standardized extracts. *J Allergy Clin Immunol* 1991; 88:43–53.
- 29 Durham SR, Walker SM, Varga EM et al. Long-term clinical efficacy of grass pollen immunotherapy. *N Engl J Med* 1999; 341:468–75.
- 30 Asero R. Effects of birch pollen-specific immunotherapy in birch pollen-hypersensitive patients. *Clin Exp Allergy* 1998; 28:1368–73.
- 31 Henzen M, Frank E, Herrmann D. Der Einfluss der Hyposensibilisierung bei Baumpollenallergie auf assoziierte Nahrungsmittelunverträglichkeit-Teil I. *Allergologie* 1994; 17:50–4.
- 32 Bolhaar STH, Tiemessen MM, Zuidmeer L et al. Efficacy of birch-pollen immunotherapy on cross-reactive food allergy confirmed by skin tests and double-blind food challenges. *Clin Exp Allergy* 2004; 34:761–9.
- 33 Möller C. Effect of pollen immunotherapy on food hypersensitivity with birch pollinosis. *Ann Allergy* 1989; 62:343–5.
- 34 Fritsch R, Bohle B, Vollmann U et al. Bet v 1, the major birch pollen allergen, and Mal d 1, the major apple allergen, cross-react at the level of allergen-specific T helper cells. *J Allergy Clin Immunol* 1998; 102:679–86.
- 35 Bohle B, Radakovics A, Jahn-Schmid B, Hoffmann-Sommergruber K, Fischer GF, Ebner C. Bet v 1, the major birch pollen allergen, initiates sensitization to Api g 1, the major allergen in celery: evidence at the T cell level. *Eur J Immunol* 2004; 33:3303–10.