

Involvement of Carbohydrate Epitopes in the IgE Response of Celery-Allergic Patients

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Key Words

Celery · Allergy · Carbohydrate epitopes · IgE · Immunoblotting

Abstract

Background: This study was performed to get further insights into antibody responses to cross-reactive carbohydrate determinants (CCD), including initial experiments to prove the biological activity of anti-CCD IgE. Earlier studies have shown that IgE specific for CCD occurs in about 25% of celery-allergic patients. The clinical significance of these antibody specificities is doubtful.

Methods: Patient sera were selected on the basis of a positive case history of celery allergy and multiple binding to high molecular weight celery allergens on immunoblots. Specific IgE to native and heated celery tuber was determined by the enzyme allergosorbent test (EAST). N-glycans were purified after extensive digestion of specific glycoproteins, such as pineapple stem bromelain, bovine fibrin, and human IgG, and used as antigens in an IgE ELISA as well as in EAST and immunoblotting inhibition experiments. Dose-related histamine release was performed with BSA neoglycoproteins containing 3–4 units of the purified glycopeptides.

Results: Seven celery-allergic patients were identified who clearly presented IgE against the N-glycan purified from bromelain which is a common structure within the

plant kingdom. Chemical defucosylation showed that α 1,3-fucose is a key structure for IgE binding. In patients with anti-CCD IgE, the maximal inhibition of celery EAST by the bromelain glycan ranged from 22 to 100%. Inhibition of celery immunoblots by preincubation of patient serum with this glycan led to a quenching of multiple bands at masses >40 kD. After linking the bromelain glycopeptide to BSA, a strong dose-related histamine release was obtained in a celery-allergic patient, occurring at lower concentrations than with the recombinant major protein allergen from celery, Api g 1. **Conclusions:** Our results demonstrate that IgE specific for CCD is common in celery-allergic patients, and can represent the major proportion of IgE against this food. α 1,3-fucose was confirmed to be an essential part of the IgE epitope. Immunoblotting inhibition indicated the presence of this carbohydrate determinant on multiple glycoproteins in celery extract. Although histamine release was only performed in 1 patient, our data show that proteins carrying multiple glycan units can be biologically active in patients sensitized to CCD.

Introduction

Alimentary allergy to plant foods is very common in birch-pollen-allergic patients [1]. This is primarily due to a family of cross-reacting major allergens which are expressed in tree pollen and many kinds of fruits, nuts and vegetables and which show a high degree of sequence identity with the major birch pollen allergen, Bet v 1.

Moreover, cross-reactivity between pollen and vegetable foods due to allergen-specific IgE that recognizes carbohydrate epitopes is a phenomenon often described in the recent literature [2]. Such 'cross-reacting carbohydrate determinants' (CCD) are present not only in a wide variety of plant extracts [3, 4], but also in material of invertebrate origin, including insect venom, caddis fly and molluscs, leading to a wide range of unexpected cross-reactivities [2–5]. The key IgE-binding epitopes in most of the described cases are α 1,3-linked fucose and β 1,2-linked xylose which occur in specific N-glycans [6]. However, the clinical significance of CCD-specific IgE antibodies is the subject of controversial discussions. Some authors [7–9] repeatedly doubted the contribution of carbohydrates to allergic symptomatology. In this context, it has been suggested that conventional allergen extracts for diagnosis or immunotherapy may be replaced by nonglycosylated recombinant allergens produced in *Escherichia coli* [10, 11]. However, studies on the clinical significance of anti-CCD IgE antibodies are rare: In 1997, van der Veen et al. [8] investigated the reactivity of IgE to CCD in grass-pollen-allergic patients with a positive peanut radioallergosorbent test (RAST) but without peanut-related symptoms and compared it with IgE from peanut-allergic patients that did not react to CCD. However, the control group of allergic patients with food-related symptoms was small: 5 peanut allergics were compared to 50 CCD-positive patients without symptoms. From this study it has been concluded that, in general, cross-reactive IgE to the glycosyl portion of glycoproteins has poor biologic activity. In contrast, our previous studies were performed in 30 patients with a positive case history of allergy to celery tuber (celeriac) who were also serologically sensitized against this food [12]. Eight of the participants clearly presented IgE specific for the above-mentioned CCD. Moreover, CCD represented the heat-stable IgE-binding material of the food, and we have shown that sera from CCD-positive patients frequently present complex IgE binding to multiple high molecular weight bands on immunoblots of plant extracts [13, 14]. This study was initiated to overcome some contrasting results in this area. Since celery belongs to the most important allergenic vegetables [1], we wanted to get further insights into the involvement

of carbohydrate epitopes in the IgE response to this food. Besides, initial histamine release tests with neoglycoproteins were performed in a celery-allergic patient to prove whether multiple CCD units on a glycoprotein may also be able to cross-link cell-bound IgE.

Materials and Methods

Human Sera

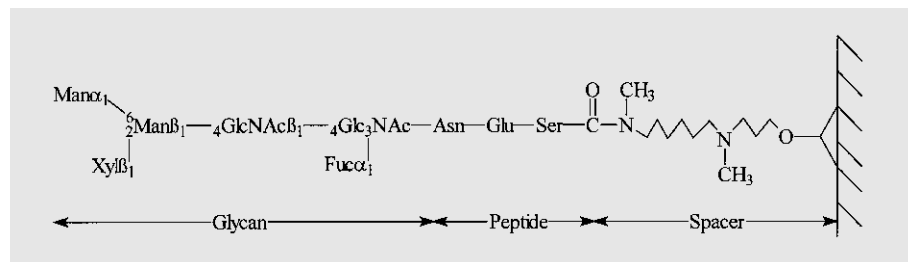
Sera from 11 adult patients were used in this study. These were chosen according to the band pattern on immunoblots and a confirmed serologic sensitization to celery tuber and birch pollen. Most of the sera (from 7 patients) were taken from participants of an earlier study [12]. Eight sera and the corresponding anamnestic data were kindly supplied by Dr. H. Aulepp (Hospital Borkum Riff, Borkum, Germany). One additional serum (PEI 41) was taken from the serum collection of the Paul Ehrlich Institute (Langen, Germany). All donors showed celery-related symptoms, reaching from the typical oral allergy syndrome to urticaria and asthma. After obtaining informed consent, sera were taken from all patients and stored at -20°C until used. One further serum from a patient with multiple inhalant and food allergies (MAST BS 103) was obtained from MAST Diagnostica (Reinfeld, Germany). The case history of celery allergy was not definitively confirmed for this patient, but the patient was allergic to several other vegetable foods. The serum from 1 nonallergic patient was included as a control. One of the celery-allergic donors, PEI 41, also participated in the histamine release assay.

Preparation and Purification of the N-Glycans

N-linked glycopeptides with the glycan structure $\text{Man}\alpha 1-6(\text{Xyl}\beta 1-2)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4(\text{Fuc}\alpha 1-3)\text{GlcNAc}$ and just 2–4 amino acid residues were purified from pineapple stem bromelain which was prepared as described [15]. Bromelain was digested twice with pronase. After the first digestion, glycopeptides were isolated by gel filtration on Sephadex G25 coarse using 1% acetic acid as the eluent. After the second pronase digestion, glycopeptides were purified on Sephadex G50 superfine with the same eluent, thereby removing any remaining larger peptides and proteins. The glycopeptide pool was subsequently subjected to two consecutive anion exchange steps using Sephadex A25 with ammonium acetate buffer for the first and sodium-borate buffer, which confers a negative charge to glycopeptides, for the second step. The glycopeptide pool was finally desalted by gel filtration and analyzed for amino acids and monosaccharides as described [4, 16, 17]. Additionally, the sample was analyzed by mass spectrometry on a DYNAMO (Thermo BioAnalysis, Santa Fe, N. Mex., USA), a linear MALDI-TOF MS with delayed extraction using 2,5-dihydroxybenzoic acid as the matrix.

Three types of controls were used: first, glycopeptides with the common pentasaccharide core $\text{Man}\alpha 1-6(\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}$ were prepared from bovine fibrin as described [3]. Second, defucosylated bromelain glycopeptides were obtained by treating bromelain glycopeptides with trifluoroacetic acid [3]. And third, glycopeptides with an α 1-6-fucose attached to the proximal GlcNAc [$\text{Man}\alpha 1-6(\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4(\text{Fuc}1-6)\text{GlcNAc-peptide}$] were isolated from human IgG by applying the same procedure. The identity of these glycopeptides was verified by the methods described above.

Fig. 1. Carbohydrate moiety isolated from bromelain covalently linked to the surface of the microtiter plate (Covalink, Nunc). A secondary amine grafted to the surface (density $10^{14}/\text{cm}^2$) serves as a 2-nm-long spacer arm.



The glycopeptides were coupled to BSA as described [3]. The resulting neoglycoproteins were found to contain an estimated amount of 3–4 mol glycopeptide per mole of protein.

A further control of the purity of the samples was carried out by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [18], followed by silver staining with 10 μg of the bromelain and the fibrin glycopeptide per lane, and 3 μg each of bromelain and of the BSA conjugates of the glycopeptides from bromelain and from defucosylated bromelain.

All glycopeptides and their BSA conjugates were stored freeze-dried at -20°C until used.

Allergen Extracts and Allergens

Recombinant major cherry allergen Pru a 1 used as an unglycosylated control peptide [19] was kindly supplied by Dr. S. Scheurer (Paul Ehrlich Institute, Langen, Germany). Recombinant major birch pollen allergen Bet v 1 [20], recombinant birch profilin (Bet v 2) [21] and recombinant Api g 1 [22], the major celery allergen, were purchased from Biomay (Linz, Austria). Recombinant major timothy grass pollen allergen Phl p 1 [23] was a kind gift from Dr. A. Petersen (Borstel Research Center, Borstel, Germany). The celery extracts were prepared at our institute from raw and heated (20 min, 100°C) celery tuber as described [12, 24]. Briefly, the plant material was shock-frosted in liquid nitrogen, homogenized in a commercial blender, extracted with 0.01 M phosphate-buffered saline (PBS), pH 7.4, dialyzed against distilled water overnight at 4°C and freeze-dried. The amount of extracted protein was estimated by a commercial dye-binding assay (Pierce, Rockford, Ill., USA). All antigens were stored freeze-dried at -20°C until used.

Total and Specific IgE

Diagnostic measurements of total and allergen-specific IgE were performed with the CAP System FEIA (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions.

For research purposes, solid phases prepared at our institute were investigated by a commercial enzyme allergosorbent test (EAST) according to the instructions of the manufacturer (Allergopharma, Reinbek, Germany). The antigens for EAST were coupled to cyanogenbromide-activated filter paper disks at concentrations depending on the source (2 $\mu\text{g}/\text{disk}$ of celery protein and 0.25 $\mu\text{g}/\text{disk}$ of the recombinant allergens, respectively) according to the method originally described by Ceska and Lundkvist [25]. The EAST results EAST were graded in arbitrary units (U/ml) by the reference system of the supplier, with a cutoff of 0.35 U/ml for positivity (EAST class 1). EAST classes are graded as follows: $0.35 < \text{class } 1 \leq 0.7$, $0.7 < \text{class } 2 \leq 3.5$; $3.5 < \text{class } 3 \leq 17.5$; $17.5 \leq \text{class } 4$. EAST classes correspond to classes of the Phadebas RAST.

Dose-related EAST inhibition studies were performed as described [14]. For inhibition assays, sera were applied at dilutions leading to about 50% of the optical density (OD) as in direct EAST (1:5 in most cases, except for the PEI 41 serum that was diluted 1:2, and the MAST BS 103 serum that was diluted 1:100).

IgE specific for carbohydrate structures was assayed by a glycan ELISA as described below. Glycopeptides were covalently linked to microtiter plates (CovalinkTM, Nunc, Wiesbaden, Germany) following the protocol of the manufacturer for immobilization of peptides using 1 μg of glycopeptide per well. Figure 1 shows the final covalently linked product composed of the glycopeptide purified from bromelain and of a secondary amine grafted to the surface of the microtiter plate which serves as bridgehead for the coupling.

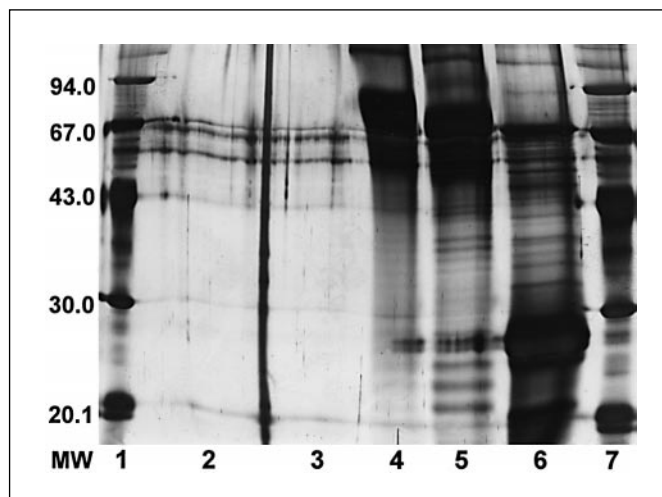
Nonspecific binding was blocked with 1% BSA in 0.05 M Tris-buffered saline (TBS), pH 7.4 for 1 h at ambient temperature. After 3 washings with TBS containing 0.05% Tween 20, the wells were incubated with 50 μl of patient serum overnight at 4°C . Bound IgE was detected by subsequent incubations with rabbit anti-human IgE (1:4,000, 1 h), goat anti-rabbit IgG biotin (1:6,000, 1 h; both from Dako, Copenhagen, Denmark) and with alkaline-phosphatase-conjugated streptavidin (1:3,000, 1 h; Caltag, San Francisco, Calif., USA). The latter three incubations were performed at room temperature, and each was followed by 3 washings as above. The washing buffer with 1% BSA was used as incubation buffer for all dilutions. After visualizing the enzymatic activity with p-nitrophenylphosphate and stopping the reaction with 1 N sodium hydroxide, ODs were read at 405 nm. The results of the glycan ELISA were judged as positive for a specific glycopeptide if the absorbance obtained with serum of a celery-allergic patient was at least twofold that of the nonallergic control ($A_p \geq 2 A_{NA}$). Results were expressed as differences of absorbance between the allergic patient and the nonallergic control ($\Delta A_p = A_p - A_{NA}$).

Electrophoresis and Immunoblotting

SDS-PAGE of celery extract was performed as described [26, 27]. After transferring the separated glycoproteins to nitrocellulose (NC) membranes by semi-dry blotting, the membranes were cut into 3 to 4-mm-wide strips and probed with sera from the allergic patients or from a nonallergic control (final dilution 1:6.7). Immunostaining of bound IgE antibodies was accomplished as described [12], with streptavidin horseradish peroxidase replaced with streptavidin alkaline phosphatase (Medac, Hamburg, Germany; 1:3,000) and 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate as the substrate.

Immunoblot inhibitions were carried out with one patient serum (MAST BS 103) diluted 1:50 and with the following inhibitors: 100 μg of celery extract or 10 μg of recombinant Api g 1 or 10 μg of

Fig. 2. Purity control of the isolated glycopeptides and their BSA conjugates by SDS-PAGE and silver staining. Lane 1: marker; lane 2: 10 µg bromelain glycopeptide; lane 3: 10 µg fibrin glycopeptide; lane 4: 3 µg BSA-coupled bromelain glycopeptide; lane 5: 3 µg BSA-coupled defucosylated bromelain glycopeptide; lane 6: 3 µg bromelain; lane 7: marker.



different glycopeptides, respectively. Blot strips with extract from celery tuber were used as the solid phase.

Histamine Release Assay

In vitro histamine release was carried out with whole blood by the method originally described by Siraganian [28, 29] and Siraganian and Brodsky [30]. Histamine was measured in a fluorescence spectrometer. Excitation was performed at 300 nm, and the emission wavelength was 450 nm. Spontaneous histamine release and the total histamine content after perchloric acid treatment were measured as controls. The allergen-induced histamine release was calculated as percent of the total amount of histamine determined after lysis of the basophils. All experiments were done at least twice.

Results

Purified Glycopeptides without Protein Contamination

The elution position of the glycopeptides on high resolution gel filtration, the differential ion exchange purifications and the data from amino acid and MALDI-MS analysis rule out any possibility of the samples being contaminated with peptide epitopes. Furthermore, SDS-PAGE silver staining (fig. 2) of 10 µg of the bromelain and the fibrin glycopeptide per lane showed no detectable protein contaminations (lanes 2 and 3). The two visible thin bands are caused by the buffer solution and occur in all other lanes, including pure sample buffer (not shown). In contrast, the protein bands of the BSA conjugates of the glycopeptides from bromelain and from defucosylated bromelain (lanes 4 and 5) and of bromelain (lane 6) were detected in the expected range between >67 kD and 22 kD, respectively. Due to the high sensitivity of the silver staining method, many impurities were detected in commercial bromelain as well as in the BSA conjugates.

Analysis of the glycopeptide isolated from pineapple stem bromelain by linear MALDI-TOF MS showed that the sample contained glycopeptides with the compositions $\text{Man}_2\text{GlcNAc}_2\text{XylFuc-AsnGlu}$ (7%), $\text{Man}_2\text{GlcNAc}_2\text{XylFuc-AsnGluSer}$ (74%) and $\text{Man}_2\text{GlcNAc}_2\text{XylFuc-AsnGluSerSer}$ (18%).

Allergen Profile of Patients Determined by Immunoblotting and EAST

As shown by figure 3, patient sera were chosen whose IgE showed multiple binding patterns on celery immunoblots. Moreover, the patients had a positive serologic IgE-dependent sensitization to celery tuber, and a positive case history of allergy to this vegetable (table 1). For comparison, 2 additional celery allergics were selected whose IgE antibodies bound to a single band with a mass of approximately 17 kD representing the major celery allergen Api g 1 which does not carry any glycosylations [22]. The IgE antibodies of 6 patients (lanes 1–6) showed major reactivities to bands >40 kD and to Api g 1 and reacted to a considerably weaker extent with additional bands <40 kD. Api g 1 was also recognized by IgE antibodies from patients Bo 98 and Bo 211 (lanes 9 and 10) who were monosensitized to this allergen. In contrast, the IgE antibodies from patients Bo 220 and Bo 172 bound to bands above and below 40 kD without recognizing Api g 1 (lanes 7 and 8). Besides this, the IgE of Bo 3, MAST BS 103, Bo 167 and Bo 220 also weakly recognized one or two additional bands at around 14 kD (lanes 1, 2, 4 and 7) which may represent celery profilin.

In the case of patients Bo 220 and MAST BS 103, sensitization to profilin was confirmed by a positive CAP to Bet v 2 (table 1). Patient Bo 82 had a positive CAP to birch pro-

Fig. 3. Binding of IgE from human sera to celery extract separated by SDS-PAGE and transferred to NC. The molecular weight corresponds to marker proteins that were stained by India ink. Bands located on the left and right side of marker are due to separated proteins of celery extract. Lanes 8–11 belong to a second run. Patient sera: lane 1: Bo 3; lane 2: MAST BS 103; lane 3: Bo 82; lane 4: Bo 167; lane 5: Bo 215; lane 6: PEI 41; lane 7: Bo 220; lane 8: Bo 172; lane 9: Bo 98; lane 10: Bo 211; lane 11: nonallergic control serum.

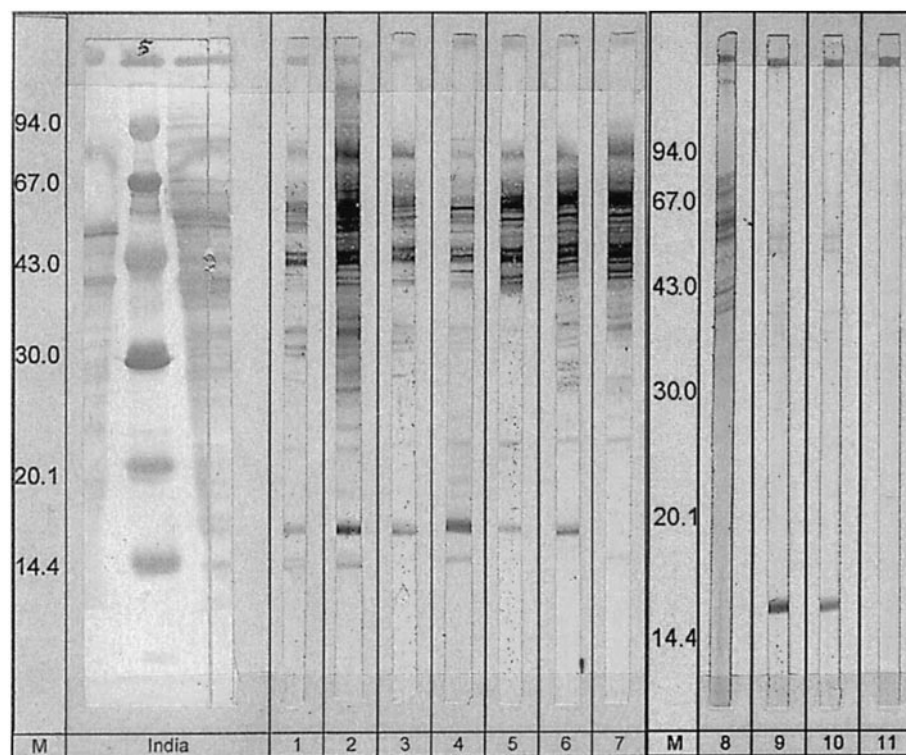


Table 1. Summary of the patient data and results of specific IgE determinations by EAST

Patient profile			EAST, U/ml ^b				
patient	IgE specific for celery allergens	celery-related symptoms	native celery	recombinant			
				Api g 1	Pru a 1	Bet v 1	Bet v 2 (CAP)
Bo3	Api g 1/CCD	OAS	1.0	1.7	27.9	21.1	0.2
Bo 82	Api g 1/CCD/ Profl	OAS, DH, V	0.7	0.3	1.5	18.4	2.7
Bo 98	Api g 1	ABP	1.3	1.6	5.1	6.5	0.2
Bo 167	Api g 1/CCD	OAS, U	1.7	0.7	22.3	21.8	0.0
Bo 172	CCD	OAS	0.6	0.0	0.6	17.9	0.0
Bo 211	Api g 1	OAS	0.5	0.7	24.4	25.9	0.2
Bo 215	Api g 1/CCD	U	1.7	2.0	25.8	21.8	0.0
Bo 220	CCD/Profl	ABP, spasm	3.9	0.0	0.0	0.0	2.0
PEI 41	Api g 1/CCD	A	1.0	0.3	0.5	2.7	0.0
MAST BS 103	Api g 1/CCD/Profl	unknown ^a	19.2	16.7	25.6	22.6	3.0

OAS = Oral allergy syndrome; DH = diarrhea; V = vomiting; ABP = abdominal pain; U = urticaria; A = asthma; Profl = profilin.

^a Multiple food allergies (spices: OAS, U).

^b Results ≥ 0.35 are positive ($\geq$ class 1).

filin but showed no IgE binding to celery extract at 14 kD. The identification of these three important allergenic structures in celery has been described in a previous investigation [14].

We used solid phases prepared at our own laboratory in the EAST for measuring the specific IgE against extract from native celery (2 μ g of protein per disk) and different recombinant allergens (0.25 μ g per disk). As can be seen in table 1, testing with extract from native celery tuber result-

ed in EAST classes from 1 to 4. Apart from patient Bo 220, all patients had IgE specific for recombinant Bet v 1 and Pru a 1, the cherry homolog to Bet v 1 and Api g 1. Pru a 1 was included in the measurements as a control for cross-reactivity studies (see EAST inhibitions). IgE specific to recombinant Api g 1 was found in all patients, except for patients Bo 172 and Bo 220. IgE specific to recombinant birch profilin was determined by CAP and was positive for the sera Bo 82, Bo 220 and MAST BS 103. All assays were negative with serum from a nonallergic patient (not shown).

IgE Binding to Glycopeptides

The patient sera were subjected to a glycan ELISA with glycopeptides (table 2) isolated from bromelain, chemically defucosylated bromelain, bovine fibrin and from human IgG covalently attached to the solid phase. All patients whose IgE recognized the high molecular weight bands in immunoblotting showed strong reactivity with the bromelain glycopeptide. The IgE of some patients also reacted with other glycopeptides, but only to a considerably weaker extent. In contrast, the IgE antibodies of patients Bo 98 and Bo 211 showed no reactivity to any of the glycopeptides.

Cross-Reactivity between Celery Extract and Carbohydrate Epitopes

To test the contribution of different celery components to the total IgE binding capacity of the whole extract, dose-dependent EAST inhibitions were carried out with a series of 10-fold dilutions of celery extract (prepared from raw or cooked vegetable), of the bromelain or fibrin glycopeptides, and of the recombinant allergens Api g 1 or Pru a 1 as inhibitors, respectively. Celery extract disks were simultaneously incubated with prediluted patient sera and the different inhibitor solutions starting with 100 µg/ml. Since three different inhibition patterns were observed in the group of participants, representative inhibition curves for the patient sera Bo 172, Bo 211 and Bo 215 are depicted in figure 4. Table 3 summarizes the results of the maximal inhibitions reached with the above-mentioned inhibitors for all sera. The specificity of the assay was demonstrated by the strong inhibition obtained with extract from raw celeriac in all cases. Moreover, recombinant Pru a 1 was taken as a negative control: apart from a high degree of sequence identity of 41% with the major celery allergen, the cherry protein Pru a 1 presented negligible cross-reactivity with IgE antibodies directed against Api g 1 [19; unpubl. data]. Indeed, rPru a 1 led to very weak inhibitions of the celery EAST in 9/10 sera. Surprisingly, in 1 case (Bo 3), a relatively strong inhibition of approximately 45% was obtained. Therefore, recombinant Phl p 1, the major allergen from timothy pollen,

Table 2. Blank-corrected absorbances measured by specific IgE ELISA using different immobilized N-linked glycopeptides

Patient	A ₄₀₅ with glycopeptide from			
	bromelain	bromelain, defucosyl	fibrin	IgG
Bo 3	1.55	1.00	–	–
Bo 83	0.99	0.41	–	0.17
Bo 98	–	–	–	–
Bo 167	0.72	0.23	0.23	–
Bo 172	1.20	–	–	0.18
Bo 211	–	–	–	–
Bo 215	1.60	–	–	–
Bo 220	2.41	–	–	–
PEI 41	2.42	–	–	–
MAST BS 103	3.00	0.33	0.19	–
– ≡ A ₄₀₅ < 0.15				

was applied as a second negative control for this serum. In contrast to Pru a 1, Phl p 1 is not related to the Bet v 1 allergen family. Phl p 1 produced a maximal inhibition <20% (table 3). The six patient sera with IgE against both Api g 1 and high molecular weight bands in immunoblotting were inhibitable in a serum-specific manner by both recombinant Api g 1 and the bromelain glycopeptide, respectively. Figure 4b shows one representative of this group of sera (Bo 215). IgE antibodies from patients Bo 98 and Bo 211 (fig. 4a) were strongly inhibited by recombinant Api g 1 and showed nearly no inhibition by the glycopeptides, whereas the opposite situation was found for the sera from patient Bo 172 (fig. 4c) and Bo 220. These two sera were uninhibitable by recombinant Api g 1, but were almost completely inhibited by the bromelain glycopeptide. Extract from heated celeriac (20 min, 100 °C) showed inhibitions above 30% only with sera with a positive glycan ELISA. This is in good correlation with reports about the deactivation of protein allergens by heating of this vegetable [13]. The sum of the maximal inhibitions of recombinant Api g 1 and the bromelain glycopeptide corresponded in most cases to the maximal inhibition reached with raw celery extract. Deviating sums obtained with some patient sera, for example Bo 167 and MAST BS 103, may be explained by additional sensitizations to IgE-binding proteins in celery extract, such as the low molecular mass bands detected by several sera in immunoblotting, which was not available for inhibition experiments. As expected from the results of the glycan ELISA, only weak maximal inhibitions were reached by the fibrin glycopeptide.

Table 3. Results of EAST inhibition experiments using extract from native celery tuber as solid phase antigen

Patient	Sensitization to	Maximal inhibition, %						
		celery extract		glycopeptide		recombinant		
		raw	cooked	bromelain	fibrin	Api g 1	Pru a 1	Phl p1
Bo3	Api g 1/CCD	80.5	42.7	31.9	19.0	54.1	45.1	17.5
Bo 82	Api g 1 /CCD/ Profl	81.4	74.4	54.6	26.6	21.3	5.0	n.d.
Bo 98	Api g 1	85.3	16.9	1.2	1.6	63.2	13.3	n.d.
Bo 167	Api g 1/CCD	85.2	17.5	21.5	0.0	39.1	7.1	n.d.
Bo 172	CCD	90.6	95.1	100.0	12.4	0.0	1.3	n.d.
Bo 211	Api g 1	93.8	23.9	5.9	13.1	89.9	19.4	n.d.
Bo 215	Api g 1/CCD	70.5	38.5	42.8	2.0	23.2	0.0	n.d.
Bo 220	CCD/Profl	80.9	83.4	91.6	6.6	0.0	0.3	n.d.
PEI 41	Api g 1/CCD	91.3	62.5	81.3	25.0	17.5	0.0	n.d.
MAST BS 103	Api g 1/CCD/Profl	75.7	64.3	47.1	0.0	4.3	18.2	n.d.

n.d. = Not determined; Profl = profilin.

Cross-reactivity between purified CCD and celery extract was further investigated by blot inhibition. The immunoblot inhibition results with serum from patient MAST BS103 are presented in figure 5. Strips coated with extract from native celery tuber were simultaneously incubated with patient serum (diluted 1:50) and different inhibitors. The IgE antibodies of the allergic patient without inhibitor bound both to bands at >40 kD, at 17 and at 14 kD (lane 1). With 100 µg of protein from native celery as inhibitor, a complete reduction of the bands could be reached (lane 4). 10 µg of recombinant Api g 1 as inhibitor exclusively quenched the 17-kD band (lane 5). No inhibition at all occurred with 10 µg of the fibrin glycopeptide as inhibitor (lane 3), but nearly all bands >40 kD were strongly reduced after application of 10 µg of the bromelain glycopeptide as inhibitor (lane 2).

Histamine Release Assay

Histamine release assays were performed in one of the patients (PEI 41). Dose-related release curves obtained after stimulation with different antigens are depicted in figure 6. Since PEI 41 is a birch-pollen-allergic patient, a relatively strong histamine release (35%) by recombinant Bet v 1 can be seen even at a very low allergen concentration (1 ng/ml). With approximately 40% histamine release in the highest applied concentrations, permanently increasing curves were obtained with celery extract and recombinant Api g 1, starting from zero levels at 10 and 100 ng/ml, respectively. No histamine release occurred with uncoupled glycopeptides (not shown) and with the BSA conjugates of the defucosylated bromelain and fibrin glycopeptides. In contrast, a con-

stant histamine release of around 18% at higher concentrations, and a maximum of 25% release at 10 ng/ml appeared with the neoglycoprotein obtained by coupling the bromelain glycopeptide to BSA. Interestingly, celery extract and recombinant Api g 1 showed no release activity at this concentration. Pure BSA (10 µg/ml) used as a negative control did not lead to any histamine release at all (not shown).

Discussion

Owing to contradictory results reported in the recent literature [8, 12], there is a demand for further research focused on IgE directed against CCD. Since more than 25% of our celery-allergic patients had IgE specific for the carbohydrate moiety of bromelain [12], this study was undertaken to obtain more detailed insights into the specificity and relevance of anti-CCD IgE in plant-food-allergic patients using celery allergy as a model.

The sera from 8 patients were initially selected on multiple immunoblot patterns that have been shown to be characteristic for patients with IgE against CCD [12, 13]. A further inclusion criterion was food allergy to celery tuber: all patients had a positive case history of type I allergy against this food, with the exception of the donor MAST BS 103 with an unknown history of celery allergy, but a known allergy against spices which frequently affects celery-allergic patients [1]. The sera from 1 nonallergic control person and from 2 celery-allergic patients whose IgE antibodies exclusively recognized the non-glycosylated major allergen Api g 1 on immunoblots were included as controls. As shown by

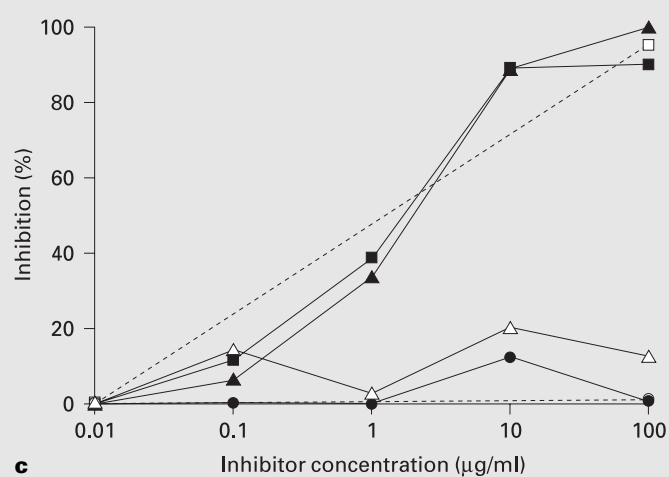
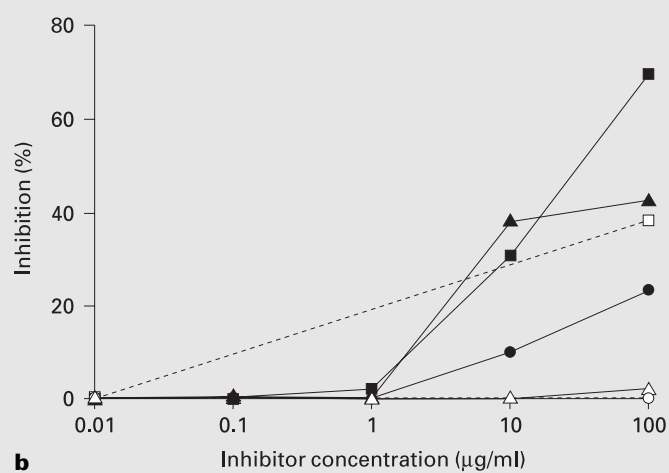
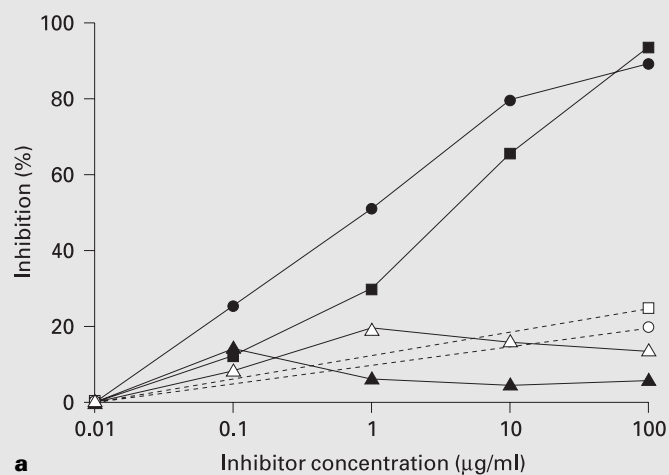


Fig. 4. Dose-related EAST inhibitions with sera from patients. **a** Bo 211. **b** Bo 215. **c** Bo 172 (all diluted 1:5). Solid phase: celery extract. Inhibitors: extract from raw celeriac (■), extract from cooked celeriac (□), glycopeptides from bromelain (▲) or fibrin (△), recombinant Api g 1 (●) or Pru a 1 (○).

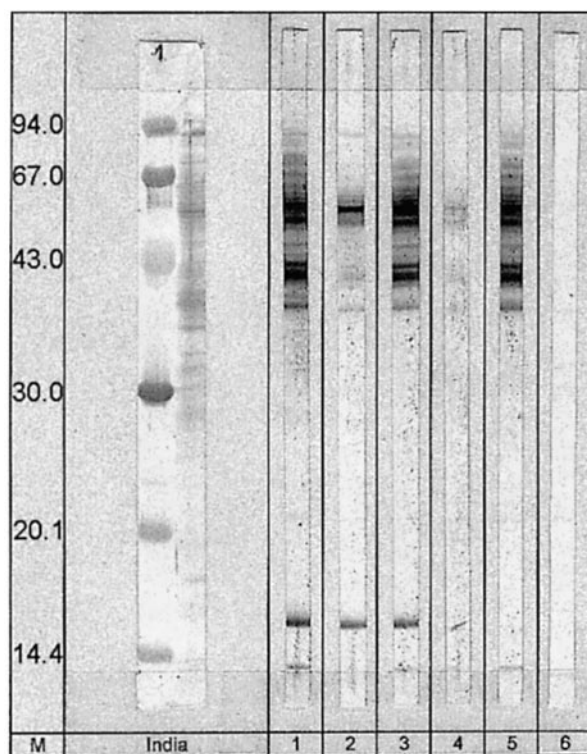


Fig. 5. Blot inhibitions with NC strips coated with extract from native celeriac and probed with human serum MAST BS 103 (diluted 1:50). The molecular weight corresponds to marker proteins that were stained by India ink. Bands located on the right side of marker are due to separated proteins of celery extract. Inhibitors: lane 1: without inhibitor; lane 2: 10 µg bromelain glycopeptide; lane 3: 10 µg fibrin glycopeptide; lane 4: 100 µg of protein from celery extract (raw); lane 5: 10 µg recombinant Api g 1; lane 6: buffer control.

the results of an IgE ELISA with purified glycopeptides covalently attached to microtiter plates, the multiple blot patterns seen in 8 of the patients correlated well with the presence of IgE specific for the N-glycan of bromelain. However, 6 of these 8 patients had additional IgE against the nonglycosylated major allergen Api g 1, 4 showed additional but weak IgE binding to a protein in the range of 14 kD. Thus, it is difficult to draw conclusions about the clinical significance of sensitizations against individual allergens from such immunoblot patterns.

The results of the IgE determination by direct EAST corresponded well with immunoblotting. All sera had a positive signal with raw celeriac, and sensitization to Api g 1 was confirmed by EAST with the recombinant molecule in 6/8 patients recognizing this band on immunoblots. Two patients with a weak EAST result of 0.3 U/ml to Api g 1 (Bo 82, PEI 41) clearly recognized this allergen on immunoblots (fig. 3). This discrepancy is most probably due

to different sensitivities of the two methods. However, in the case of PEI 41, sensitization to Api g 1 was confirmed by basophil histamine release. Since the birch pollen allergen Bet v 1 is thought to initiate sensitization to the related allergens in foods, the strong IgE binding to this allergen may reflect this situation. Birch pollen profilin (Bet v 2) was applied as an indicator for sensitization against the panallergen profilin which is present as a cross-reactive allergen in many kinds of plant foods [31]. The data show that profilin is of minor importance in our group of patients. Only sera from 3 patients had a positive CAP to Bet v 2. Patient Bo 82 with a positive CAP to Bet v 2 did not recognize a band at 14 kD on celery immunoblots. This observation may be explained by a partial cross-reactivity between these related allergens and the recognition of a non-cross-reactive epitope on Bet v 2 by IgE from this patient.

In the glycan ELISA, purified CCD-containing glycopeptides of known carbohydrate and amino acid composition were used as solid phase antigens. The glycopeptides contain 2–4 amino acid residues, a size that is below that of published sequence epitopes which cover at least 6 amino acid residues. These small peptides are bound to the carbohydrate moiety by an N-glycosidic linkage via Asn. Since no larger peptidic structures were present in the preparations, there are no protein epitopes but only CCD epitopes responsible for IgE binding in the glycan ELISA. IgE from 8 food-allergic patients reacted strongly with a specific glycopeptide containing α 1,3-linked fucose, whereas the IgE from the nonallergic control and from the 2 celery-allergic patients who are exclusively sensitized to Api g 1 showed no reaction to any of the glycopeptides. The participation of the Asn-linked peptide backbone in the epitope can largely be excluded, since defucosylated control glycopeptides that carry the same Asn-linked peptides and other control Asn-linked glycopeptides did not react with IgE from most of the patients. However, the 3-residue peptide backbone which was covalently bound to the surface of the microtiter wells may participate in minor interactions with the binding site of the antibodies or could be necessary to maintain a native conformation of the glycan (fig. 1).

At the serological level, the results of EAST inhibition experiments permit an estimation of the proportion of IgE directed against individual allergenic structures present in celery extract: for the 2 patients who appeared to be almost exclusively sensitized to CCD-containing glycoproteins of celery extract (Bo 172, Bo 220), the inhibitory activity of celery extract was completely mimicked by the bromelain glycan, whereas Api g 1 had no inhibitory activity. In contrast, Api g 1 and native celery represented the only relevant inhibitors in patients Bo 98 and Bo 211 who showed

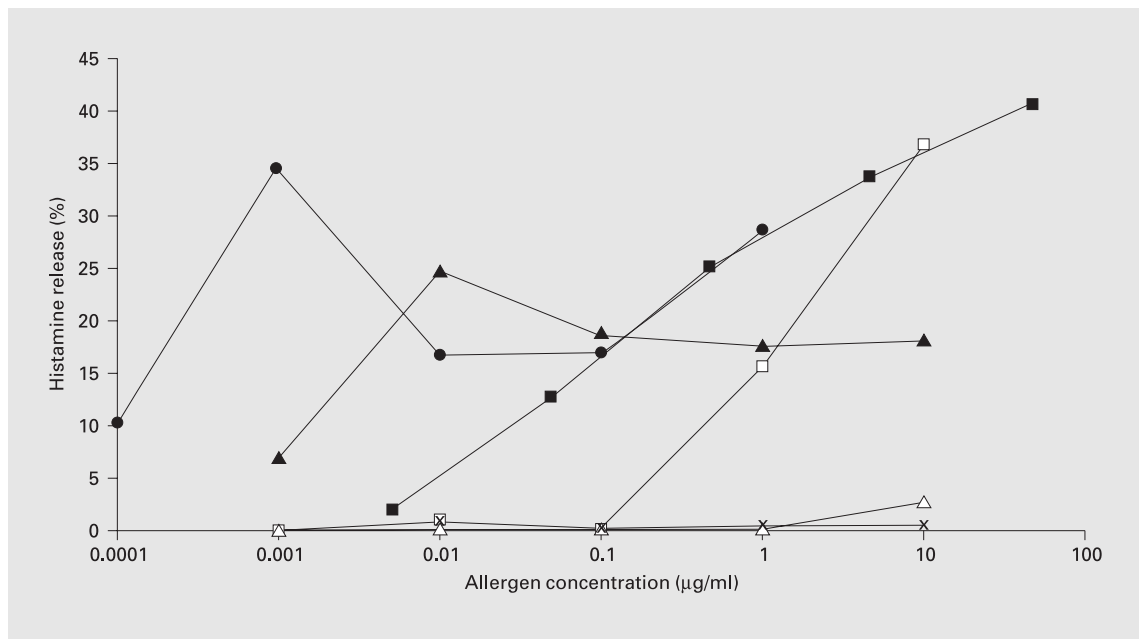


Fig. 6. Histamine release assay with whole blood from patient PEI 41. Dose-related curves were obtained by stimulations with the following allergens: extract from raw celery tuber (■), BSA conjugates of glycopeptides from bromelain (▲), defucosylated bromelain (△) and fibrin (⊠), with recombinant Api g 1 (□) and recombinant Bet v 1 (●).

monosensitization to the major allergen on immunoblots. These results demonstrate firstly the specificity of the allergen identification performed by immunoblotting and ELISA, and secondly that CCD can be the only IgE-binding material in celery extract for patients with food-related symptoms. In the 6 patients with IgE against both Api g 1 and CCD, the maximal inhibition with the bromelain glycan exceeded the maximal level obtained with Api g 1 in 4 cases. Since the inhibition graphs indicated that the amount of inhibitor antigens was in the saturation range, we conclude that CCD can bind a major proportion of IgE in a relevant number of celery-allergic patients. In 2 patients (Bo 167, MAST BS 103), the sum of maximal inhibitions obtained with Api g 1 and the bromelain glycan was more than 20% below the maximum obtained with extract from raw celery tuber. This effect may be due to an additional antibody population against an additional protein in the range of 14 kD which was weakly recognized by IgE from these patients on celery immunoblots. Since immunoblot inhibitions with celery profilin or rBet v 2 were not performed, it remains unclear whether this 14-kD band represents the known celery allergen profilin. The inhibition potency obtained with extract from cooked celeriac correlated well with the inhibition by bromelain glycan with maximal inhibitions <25%

in the 2 patients monosensitized to Api g 1. This may underline the importance of CCD as the heat-stable IgE-binding component of celery, and the heat lability of the major allergen [12, 14].

The cherry protein Pru a 1 was applied as an inhibition control. The high degree of structural similarity of this allergen to Bet v 1 [19] is reflected by a similar degree of IgE reactivity to both allergens in EAST for most of the sera. Apart from the high proportion of specific IgE in the sera, preincubation with Pru a 1 had little effect on the IgE binding to Api g 1 in the inhibition assay, confirming the existence of unrelated epitopes on these allergens [19]. Only one serum (Bo 3) showed a relatively strong inhibition by recombinant Pru a 1 (45%). Since this could be due to similar epitopes of Api g 1 and Pru a 1 recognized by IgE from this specific patient, we used recombinant Phl p 1, the major allergen from timothy pollen, as a second negative control for this serum. Phl p 1 produced a maximal inhibition of 17.5%, which is below our cutoff for specific binding.

Since pineapple stem bromelain glycans strongly cross-reacted with IgE directed against celery allergens, our inhibition data confirm the high degree of cross-reactivity of these epitopes. Moreover, blot inhibition with the purified glycopeptide proved that identical carbohydrate determi-

nants can occur on multiple high molecular weight glycoproteins from one plant species. Taken together, these results correspond well with reports describing the ubiquitous occurrence of CCD containing fucose and/or xylose in pollens of olive tree, Japanese cedar, Bermuda grass, and in glycoproteins of celery, tomato, potato, bean, soybean and pea [2, 6, 7].

Many glycoproteins such as the cysteine proteinase bromelain possess only one N-linked glycan [32]. Such a monovalent epitope would not be able to induce cross-linking of mast-cell-bound IgE if the antibody response was restricted to CCD. However, other glycoproteins with more than one glycan unit, e.g. horseradish peroxidase with 8 N-linked glycans [33], would be candidates for inducing a mediator release.

To obtain initial data on the potential of proteins containing multiple repeats of identical glycans to induce type I allergic reactions, histamine release assays were performed on a whole blood sample from one participant with IgE against the nonglycosylated major allergens of birch pollen and celery, as well as against CCD. As expected, the uncoupled glycopeptides as monovalent epitopes were unable to induce a mediator release. The strongest mediator release from IgE-sensitized basophils was obtained after stimulation with Bet v 1 which is known to be a very potent allergen [34]. Interestingly, a similarly shaped release curve with an about tenfold reduced potency resulted from stimulation by a BSA neoglycoprotein containing 3–4 bromelain glycans (fig. 6). This construct with polyvalent epitopes showed a lower maximal release than Bet v 1, Api g 1 and celery extract, which may reflect qualitative differences in the allergenicity between the CCD and the protein allergens. Nevertheless the neoglycoprotein showed a remarkable activity with a maximal release of nearly 25%, whereas 100-fold higher amounts of Api g 1 were necessary to trigger the same degree of cell degranulation. In contrast, pure BSA without the coupled carbohydrate moiety did not lead to histamine release, even at the maximal concentration of 10 µg/ml. Since this assay is based on the essential event of the allergic reaction, our results support the hypothesis that proteins with multiple glycosylations may possess a biologic activity which is related to the carbohydrate moieties, and that this activity can be similar to the potency of allergens with protein epitopes. A recent study that was performed on the carbohydrate moiety of the major olive pollen allergen, Ole e 1, supports our finding that IgE specific to CCD can possess a high biological activity [35].

Since all patients possess IgE specific either for rBet v 1 or rBet v 2, i.e. for epitopes of proteins without glycosyla-

tions, the conclusions on the clinical relevance of IgE to CCD still remain difficult. However, the inhibition results proved that in the case of celery allergy, for some patients the only detectable IgE directed against celery extract was specific for CCD, and that IgE specific for protein allergens from birch pollen in these sera did not cross-react with related celery proteins.

In summary, we conclude that glycoproteins carrying complex N-glycans are important IgE-reactive structures for celery-allergic patients, with α 1,3-linked fucose being more important for maintaining the antibody reactivity than β 1,2-linked xylose. There is evidence that the clinical symptoms of celery allergy elicited in our patients are not only attributed to proteins without glycosylations such as the major allergen Api g 1, but also to carbohydrate epitopes. This view is supported by the observations that (1) CCD appeared to bind the highest percentage of specific IgE in more than 50% of patients with this specific sensitization, (2) that patients were identified whose only detectable IgE directed against celery was specific for CCD without any additional recognition of protein allergens of celery, and (3) that a strong dose related mediator release in the same concentration range as with protein allergens can be attributed to carbohydrate epitopes indicating the potential of biological activity in this assay system. Unfortunately, for practical reasons, the histamine release assay was performed only in 1 patient, but not in the whole group of participants. Investigations of the mediator release in an extended group of allergic patients following our strategy are required to obtain more quantitative data about the clinical significance of anti-CCD responses in type I allergic diseases. The results of such studies will be important for defining future demands on recombinant allergens for in vivo applications.

There is an increasing trend for using recombinant allergens in the diagnosis and therapy of type I allergic reactions [10, 11]. Almost all well-characterized recombinant allergenic proteins which have so far been applied in humans were derived from transformed *E. coli* and thus lack glycosylations and other posttranslational modifications of their natural eucaryotic counterparts. Provided that these bacterial proteins can be obtained in the native conformation and CCD would indeed be without clinical significance in all patients [8], recombinant allergens would offer a lot of advantages and would be the ideal diagnostic and therapeutic material. Standardization in mass units would be easy, and false-positive serologic assays caused by the presence of CCD in natural extracts would be excluded. On the contrary, if IgE against CCD were able to trigger allergic symptomatology at least in a significant subgroup of patients sen-

sitized against these determinants, important IgE specificities would be overlooked by the existing recombinants. In this case, additional diagnostic tests such as the CCD-ELISA applied in this study would be required.

For some allergens, recent results point at the latter case. For example, the IgE-binding capacity of the recombinant allergens Phl p 1 and Phl p 5, expressed in *E. coli*, was significantly lower than that of natural Lol p 1 and Lol p 5 [36]. A possibility to overcome the problem of incorrect folding and lacking glycosylation occurring with the procaryotic expression system is the use of eucaryotic expression systems. For Ves v 5 [37], Cyn d 1 [38], Der p 1 [39] and hyaluronidase [40], successful trials in that respect were described. In all of these cases, the recombinant proteins expressed in yeast [37–39] or in baculovirus-infected insect cells (BIC) [40] possessed a nearly native conformation, and the biological activity was similar to their native counterparts in contrast to the recombinant proteins produced in *E. coli*. So, the latter were considered to be candidates for

evaluation as therapeutic agents because of their weak or non-IgE binding, whereas the IgE-reactive recombinant yeast or BIC proteins are better suited as diagnostic tools [38]. To get further insights into the biological relevance of IgE against carbohydrate epitopes, it would be interesting to investigate whether BIC-expressed high molecular weight celery allergens are active in patients allergic to this vegetable and presenting IgE specific for CCD.

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References

- Jäger L, Wüthrich B: Nahrungsmittelallergien und -intoleranzen, ed 1. Stuttgart, Fischer, 1998.
- Aalberse RC, van Ree R: Cross-reactive carbohydrate determinants. Clin Rev Allergy Immunol 1997;15:375–387.
- Wilson IBH, Harthill JE, Mullin NP, Ashford DA, Altmann F: Core α 1,3-fucose is a key part of the epitope recognized by antibodies reacting against plant N-linked oligo-saccharides and is present in a wide variety of plant extracts. Glycobiology 1998;8:651–661.
- Wilson IBH, Altmann F: Structural analysis of N-glycans from allergenic grass, ragweed and tree pollens: Core α 1,3-fucose and xylose present in all pollens examined. Glycoconj J, in press.
- Tretter V, Altmann F, Kubelka V, März L, Becker WM: Fucose α 1,3-linked to the core region of glycoprotein N-glycans creates an important epitope for IgE from honeybee venom-allergic individuals, Int Arch Allergy Immunol 1993;102:259–266.
- Vieths S: Allergenic cross-reactivity, food allergy and pollen. Environ Toxicol Pharmacol 1997;4:61–70.
- Aalberse RC, van Ree R: Cross-reactive carbohydrate determinants; Wüthrich B, Ortolani C (eds): in Highlights in Food Allergy. Monogr Allergy Basel, Karger, 1996, vol 32 pp 78–83.
- Van der Veen MJ, Van Ree R, Aalberse RC, Akkerdaas J, Koppelman SJ, Jansen, HM, Van der Zee JS: Poor biologic activity of cross-reactive IgE directed to carbohydrate determinants of glycoproteins. J Allergy Clin Immunol 1997;100:327–334.
- Aalberse RC: Clinical relevance of carbohydrate allergen epitopes, Allergy 1998;53:54–57.
- Valenta R, Laffer S, Vrtala S, Gronlund H, Elfman L, Sperr WR, Valenta P, Ferreira F, Mayer P, Liehl E, Heiss S, Steiner R, Eichler HG, Sugani M, Kraft D: Recombinant allergens. Steps on the way to diagnosis and therapy of type I allergy. Adv Exp Med Biol 1996;409:185–196.
- Valenta R, Vrtala S, Laffer S, Spitzauer S, Kraft D: Recombinant allergens. Allergy 1998;53:552–561.
- Jankiewicz A, Aulepp, H, Altmann F, Fötisch K, Vieths S: Serological investigation of 30 celery-allergic patients with particular consideration of the thermal stability of IgE binding celery allergens. Allergo J 1998;7:87–95.
- Vieths S, Jankiewicz A, Wüthrich B, Baltes W: Immunoblot study of IgE binding allergens in celery roots. Ann Allergy 1995;74:48–55.
- Jankiewicz A, Baltes W, Bögl KW, Dehne LI, Jamin A, Hoffmann A, Haustein D, Vieths S: Influence of food processing on the immunological stability of celery allergens. J Sci Food Agric 1997;75:359–370.
- Tretter V, Altmann F, März L: Peptide-N⁴-(N-acetyl- β -glucosaminyl) asparaginase amidase F cannot release glycans with fucose attached α 1 $\rightarrow$ 3 to the asparagine-linked N-acetylglucosamine residue. Eur J Biochem 1991;199:647–652.
- Altmann F: Determination of amino acid sugars and amino acids in glyco-conjugates using pre-column derivatization with o-phthalaldehyde. Anal Biochem 1992;204:215–219.
- Fu D, O'Neill RA: Monosaccharide composition analysis of oligosaccharides and glycoproteins by high-performance liquid chromatography. Anal Biochem 1995;227:377–384.
- Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–685.
- Scheurer S, Metzner K, Haustein D, Vieths S: Molecular cloning, expression and characterization of Pru a 1, the major cherry allergen. Mol Immunol 1997;34:619–629.
- Breiteneder H, Pettenburger K, Bito A, Valenta R, Kraft D, Rumpold H, Scheiner O, Breitenbach M: The gene encoding for the major birch pollen allergen, *Betv1*, is highly homologous to a pea disease resistance response gene. EMBO J 1989;8:1935–1938.
- Valenta R, Duchene M, Pettenburger K, Sillaber C, Valent P, Bettelheim P, Breitenbach M, Rumpold H, Kraft D, Scheiner O: Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. Science 1991;253:557–560.
- Breiteneder H, Hoffmann-Sommergruber K, O'Riordain G, Susani M, Ahorn H, Ebner C, Kraft D, Scheiner O: Molecular characterization of Api g 1, the major allergen of celery (*Apium graveolens*), and its immunological and structural relationships to a group of 17 kDa tree pollen allergens. Eur J Biochem 1995;233:484–489.
- Petersen A, Schramm G, Bufe A, Schlaak M, Becker W-M: Structural investigations of the major allergen *Phl p I* on the complementary DNA and protein level. J Allergy Clin Immunol 1995;95:1–8.

- 24 Vieths S, Schöning B, Brockmann S, Aulepp H: Untersuchungen zur Allergie gegen Lebensmittel pflanzlicher Herkunft: Herstellung und Charakterisierung von Obst- und Gemüseextrakten für serologische Untersuchungen. Dtsch Lebensm Rundsch 1992; 88:239–243, 272–279.
- 25 Ceska M, Lundkvist U: A new and simple radioimmunoassay method for the determination of IgE. Immunochemistry 1972;9:1021–1030.
- 26 Vieths S, Mayer M, Baumgart M: Allergy to fruits and vegetables in pollen-sensitive patients: Allergen characterization by IgE immunoblotting and peroxidase staining. Food Agric Immunol 1992b;4:181–197.
- 27 Vieths S, Mayer M, Baumgart M: Food Allergy: Specific binding of IgE antibodies from plant food sensitized individuals to carbohydrate epitopes. Food Agric Immunol 1994; 6:453–463.
- 28 Siraganian RP: An automated continuous-flow system for the extraction and fluorometric analysis of histamine. Anal Biochem 1974;57: 383–394.
- 29 Siraganian RP: Refinements in the automated histamine analysis system. J Immunol Methods 1975;7:283–290.
- 30 Siraganian RP, Brodsky MJ: Automated histamine analysis for in vitro allergy testing. I. A method utilizing allergen-induced histamine release from whole blood. J Allergy Clin Immunol 1976;57:525–540.
- 31 Van Ree R, Voitenko V, Van Leeuwen WA, Aalberse RC: Profilin is a cross-reactive allergen in pollen and vegetable foods. Int Arch Allergy Immunol 1992;98:97–104.
- 32 Yasuda Y, Takahashi N, Murachi T: The composition and structure of the carbohydrate moiety of the stem bromelain. Biochemistry 1970; 9:25–32.
- 33 Welinder KG: Amino acid sequence studies of horseradish peroxidase. Amino and carboxyl termini, cyanogen bromide and tryptic fragments, the complete sequence, and some structural characteristics of horseradish peroxidase C. Eur J Biochem 1979;96:483–502.
- 34 Vieths S, Janek K, Aulepp H, Petersen A: Isolation and characterization of the 18-kDa major apple allergen and comparison with the major birch pollen allergen (*Bet v 1*). Allergy 1995;50:421–430.
- 35 Batanero E, Crespo JF, Monsalve RI, Martin-Esteban M, Villalba M, Rodriguez R: IgE-binding and histamine release capabilities of the main carbohydrate component isolated from the major allergen of olive tree pollen, Ole e 1. J Allergy Clin Immunol 1999;103: 147–153.
- 36 Van Ree R, van Leeuwen WA, Aalberse RC: How far can we simplify in vitro diagnostics for grass pollen allergy? A study with 17 whole pollen extracts and purified natural and recombinant major allergens. J Allergy Clin Immunol 1998;102:184–190.
- 37 Monsalve R, Lu G, Agosto R, King TP: Recombinant yellow jacket venom allergen, antigen 5, with native conformation of the natural allergen (abstract). J Allergy Clin Immunol 1998;101(1–2):5200.
- 38 Smith PM, Suphioglu C, Griffith IJ, Theriault, K, Knox B, Singh MB: Cloning and expression in yeast *Pichia pastoris* of a biologically active form of Cyn d 1, the major allergen of Bermuda grass pollen. J Allergy Clin Immunol 1996;98:331–343.
- 39 Chua K-Y, Kehal PK, Thomas WR, Vaughan PR, Macreadie IG: High frequency binding of IgE to the *Der p* allergen expressed in yeast. J Allergy Clin Immunol 1992;89:95–102.
- 40 Soldatova LN, Crameri R, Gmachl M, Kemeny DM, Schmidt M, Weber M, Mueller UR: Superior biologic activity of the recombinant bee venom allergen hyaluronidase expressed in baculovirus-infected insect cells as compared with *Escherichia coli*. J Allergy Clin Immunol 1998;101:691–698.

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