

Lack of crossreaction with Bet v 1 in patients sensitized to Dau c 1, a carrot allergen

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Background: Pollen-related food allergies to fresh fruit and vegetables are a well-known clinical phenomenon. Allergens related to Bet v 1 are responsible for these cross-reactions.

Objective: To characterize the allergen recognized by four carrot-allergic patients.

Methods: Sera from four patients showing strong immediate systemic reactions after contact or ingestion of raw carrot were studied by immunoblotting. The 18-kD allergen, named Dau c 1, was isolated by ethanol precipitation and specific extraction after SDS-PAGE and its N-terminal amino acid sequence was determined.

Results: All the patients had significant levels of specific IgE to carrot, but no specific IgE to birch pollen was detected in any of them. IgE immunodetection with the sera only recognized a single band of around 18 kD in raw carrot and in celery (with weaker reaction). No reactive band was found with birch pollen. These results were confirmed using a polyclonal anti-carrot antiserum. The carrot IgE-binding protein had a pI of 4.2 and its N-terminal sequence was homologous to that of Bet v 1 and to allergens previously described in celery and other foods. The four patients studied were not sensitized to birch pollen and three of them tolerated fruit ingestion.

Conclusion: The whole study indicated that a sensitization to Dau c 1 induces IgE antibodies that do not cross-react with birch pollen allergens.

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INTRODUCTION

Intolerance reactions after ingestion of apple, hazelnut, and other fruits as well as celery and carrot, are often found in birch pollen-allergic patients.¹⁻⁴ This clustering of hypersensitivities exceeds

the phylogenetic relationships between botanical families and is currently explained by cross-reactions between homologous allergens present in all these plant materials.^{5,6} A family of 16 to 18-kD proteins includes the major allergen from birch (Bet v 1), hazel (Cor a 1), and alder (Aln g 1) pollens as well as the main IgE-binding polypeptides of apple (Mal d 1) and celery (Api g 1).⁷⁻¹⁰ Members of this allergen family have been recently detected in other fruits and vegetables such as cherry and carrot.¹¹⁻¹³ Furthermore, a high degree of sequence identity between these allergens and several pathogenesis-related proteins of the PR-10 group has been found.^{7,14} PR-10 are intracellular plant proteins induced in pathologic situations that seem to be ubiquitous in the plant kingdom and may exert ribonuclease activity.¹⁵ Components of this group have been detected in several species such as soybean, po-

tato, and asparagus, where their potential allergenic properties remain to be demonstrated.¹⁶

The aim of the present work was to identify the single allergen to which several patients allergic to carrot were monosensitized.¹⁴

MATERIALS AND METHODS

Isolation of Dau c 1

A crude carrot extract was obtained by mincing carrot in phosphate-buffered saline (PBS) at a 5% wt/vol. The mixture was shaken for 30 minutes, centrifuged at 4,500 *g* for 10 minutes, and the supernatant collected (crude extract). To obtain a protein preparation highly enriched in the 18-kD allergen, the crude extract was mixed 1:1 vol/vol with absolute ethanol and 1 hour later centrifuged at 4,500 *g* for 15 minutes. The pellet obtained was discarded and the amount of ethanol was raised to a final content of 75% vol/vol. One hour later the mixture was centrifuged at 4,500 *g* for 15 minutes and the pellet obtained was dissolved in PBS in 1/5 of the starting volume. The isolation of Dau c 1 was carried out from the allergen-enriched protein preparation. The sample was separated by SDS-PAGE and electrotransferred onto a polyvinylidene difluoride (PVDF) membrane as in Sánchez-Monge et al.¹⁷ After identification by Amido black staining of a narrow membrane strip, the allergen was cut off and extracted in 80% formic acid for 1 hour and then with ethanol:water:triethylamine (2:2:1) for 1 hour. The solvents were vacuum-evaporated and the isolated protein redissolved in 0.1 M ammonium acetate.

The isoelectric point of Dau c 1 was determined by isoelectrofocusing in IsoGel Agarose IEF Plates (pH 3-10)

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slab gels (FMC BioProducts, Rockland, USA) including the appropriate protein markers. N-terminal aminoacid sequencing was performed by standard methods, using a gas-phase Applied Biosystem 494 sequencer.

Polyclonal Antiserum

A rabbit antiserum to the protein preparation enriched in Dau c 1 was obtained by fortnightly subcutaneous injections of 200 µg of the antigen in saline. Freund’s complete adjuvant was used for the first injection and incomplete adjuvant was used thereafter.

Patients, Skin Prick Tests, Total and Specific IgE

The four patients included in the study had severe systemic immediate reactions after ingestion of raw carrots. Skin prick tests were performed with a standard battery including pollens from grasses, birch, mugwort, and olive. Tests were considered positive when the wheal had a diameter > 3 mm. Total and specific IgE was determined by CAP (Pharmacia-Upjohn, Sweden) according to the manufacturer’s instructions and expressed in kU/L.

SDS-PAGE and Immunoblotting

The non-reduced, non-heated samples were subjected to a 16% acrylamide SDS-PAGE and stained according to standard methods. For immunoblotting assays, the separated proteins were transferred by a diffusion method, as previously described.^{18,19} After blocking in 3% NP-40 in PBS for 30 minutes, the membranes were incubated overnight with 10 mL of a 1:20 dilution of the different sera. Thereafter,

the nitrocellulose membranes were subsequently incubated with 10 mL of a biotin-labeled monoclonal anti-IgE (3 hours at a 1:1,000 dilution), streptavidin-alkaline phosphatase (1 hour at a 1:2,000 dilution) and finally 30 minutes with the substrate (BCIP-NBT). A polyclonal rabbit antiserum to the purified protein was used in some assays. The blotting procedure described above was followed with minor modifications: the polyclonal antiserum was incubated for 1 hour at a 1:5,000 dilution and a goat anti-rabbit IgG labeled with alkaline phosphatase was used at a 1:4,000 dilution for 1 hour.¹⁸

RESULTS

The principal clinical aspects of the four patients are shown in Table 1. No clear-cut clinical symptoms suggestive of hay fever were observed in the patients, but mild rhinoconjunctivitis was present in three subjects. These three subjects had also minor reactions (oral allergy syndrome) with other fruits and vegetables (Table 1). Two of them had been unsuccessfully treated with grass pollen immunotherapy, probably based on the presence of positive skin tests to grass pollen. Based on the clinical data, specific IgE to several pollens, including olive, a frequent sensitizer in Spain, was determined (Table 2). All four patients showed high specific IgE levels to carrot, but no specific IgE to birch and only two class 1 values to mugwort were obtained. These results were confirmed by IgE immunoblotting with the sera from the four carrot-sensitized patients (Fig 1). A single

band of around 18 kD was responsible for the IgE-binding properties of the crude carrot extract in the four cases. Similar immunodetection patterns were obtained when a highly enriched antigen preparation was used. In contrast, no reactive bands were found in birch and grass pollen and only one of the sera weakly detected a single component of around 20 kD from olive pollen. Grass and olive pollen were tested for their local importance as sensitizers in Spain and birch pollen was selected due to its cross-reacting properties. No IgE-reacting bands were detected in chard, bean, apple, strawberry, peanut, or potato (data not shown).

Additional evidence supporting this lack of cross-reaction was observed when a polyclonal antiserum raised against the protein preparation highly enriched in the 18-kD antigen was tested (Fig 1 E). No specific antibodies to olive, grass, or birch pollen could be detected, in spite of the heavy recognition of the carrot allergen. Crude carrot and celery extracts were jointly assayed by blotting to check if this lack of recognition of pollen allergens was due to a drop of the affinity constant (Fig 2). Both antigens had a single IgE-binding protein of similar molecular weight, but the carrot allergen showed higher intensity.

To characterize the major carrot allergen detected in Figure 1, the protein preparation enriched in this antigen was used as starting material. In spite of the high degree of enrichment ob-

Table 1. Principal Clinical Data

Patient No.	Age (y)	Sex	Atopy	Seasonal Symptoms	Symptoms* After Ingestion of Raw Carrot	Other Foods Inducing Symptoms	Tolerance of Other Apiaceae
1	40	Female	Yes	RC	RC-Asthma	Chard, Bean	Yes
2	39	Female	Yes	RC	OAS	Apple, Cherry, Strawberry, Peanut	No
3	34	Female	No	No	RC-U-AEG	No	Yes
4	33	Female	Yes	RC	RC-AEG	Potato	Yes

* RC = Rhinoconjunctivitis, U = urticaria, AEG = angioedema, and OAS = oral allergy syndrome.

Table 2. Total IgE, Specific IgE and Skin Prick Tests

	1	2	3	4
Carrot	27	15	42	8
Grass pollen	<0.35 (+)	<0.35 (-)	3.29 (+)	<0.35 (+)
Olive pollen	<0.35 (-)	2.52 (+)	11.41 (+)	<0.35 (+)
Mugwort pollen	<0.35 (-)	0.37 (-)	0.41 (-)	<0.35 (-)
Birch pollen	<0.35 (-)	<0.35 (-)	<0.35 (-)	<0.35 (-)
Total IgE	811	407	411	191

* The results of the skin tests to the different allergens are shown in brackets.

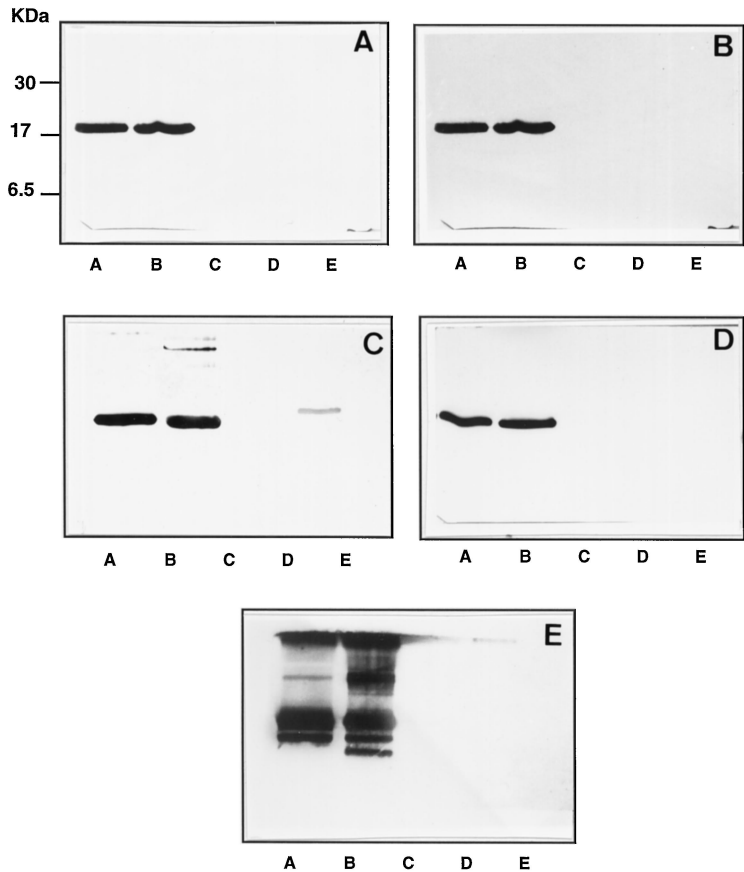


Figure 1. IgE-immunoblot of the four patients' sera (panels A–D) to raw carrot extract (lane A), Dau c 1 enriched fraction (lane B), birch pollen (lane C), olive pollen (lane D), and grass pollen mixture (lane E). The rabbit polyclonal antiserum is used in panel E with the same antigen distribution.

tained, as shown by silver staining, both the rabbit antiserum and the human sera detected the presence of contaminants (Fig 3). The allergen was therefore further purified by SDS-PAGE separation and selective elution of the corresponding band. The purified protein showed a single band of 18 kD and a pI of 4.2 and reacted with the human sera and the rabbit antiserum

(Fig 3). N-terminal amino acid sequencing gave no heterogeneity in any of the determined residues. The sequence obtained, as well as its comparison with several members of the Bet v 1 allergen family is summarized in Fig 4.

DISCUSSION

The present study included four female patients with clear cut symptoms after

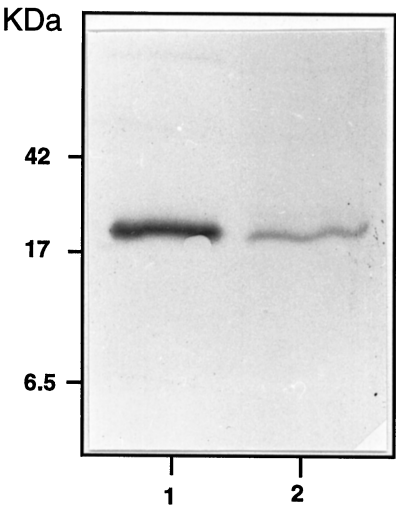


Figure 2. Specific IgE-immunoblot to carrot extract (lane 1) and celery extract (lane 2) using a serum pool.

contact or ingestion of raw carrot. Three of them had also sporadic mild rhinoconjunctivitis during the spring months. All of them tolerated the ingestion of cooked carrots, a fact that suggested the thermolability of the allergen, and did not remember any problems with celery, but celery is rarely consumed in Spain. In this group of patients other foods causing oral allergy syndrome were chard, bean, apple, cherry, strawberry, peanut, and potato. No allergens were detected in these vegetables when studied by immunoblotting. The specific IgE determination left no doubt about the sensitization to carrot and the immunodetection clearly showed that a single component of around 18 kD of the raw extract bound IgE, as previously shown by us.¹⁸ The purification and characterization of this allergen showed that it belonged to the Bet v 1 family and the results were further supported by the N-terminal amino acid sequence. No specific IgE to grass or birch pollen was obtained in the IgE-immunoblotting and only faint recognition of a protein around 20 kD in olive pollen was found in a patient. Olive pollen is a frequent sensitizer in Spain, but no cross-reaction with birch pollen has yet been described. A pool of the human sera detected a homolo-

gous allergen in celery, but the less intense recognition of a single allergen in this vegetable was probably the result of a lower affinity constant due to variations in the amino acid sequence (Fig 4). Although similar allergens were not detected by these sera or by the polyclonal antiserum in bean, chard, peanut, apple, potato, or cherry, PR-proteins with related sequences have been described in some of these fruit and vegetables.¹⁵ Interestingly, according to Yamamoto et al,¹³ when a phylogenetic tree was constructed using the amino acid sequences of the different members of the Bet v 1 family so far reported, they could be classified into four groups. Dau c 1 belongs to the group I, which also includes Api g 1 and the parsley PR1-1 and 1-3 proteins, being the rest of Bet v 1 related proteins classified in the other groups.

Breiteneder et al¹⁰ suggest that allergy to celery is an epiphenomenon due to a sensitization by inhalation of the cross-reacting pollen allergens (Bet v 1) in the first place. This interesting theory could explain why patients become sensitized to food allergens. Asero²⁰ recently describes that in patients with birch pollen sensitivity, those having oral allergy syndrome due to Apiaceae hypersensitivity have the highest birch pollen-specific IgE levels. In this context, our birch pollen negative carrot-sensitized patients seem to be an exception. Birch trees are rare in Spain, therefore our patients can not be sensitized to Bet v 1 by inhalation. This fact could explain the lack of specific IgE to birch pollen. Similar negative results to birch and mugwort pollens have been described by others in Spanish carrot-allergic patients.^{21,22} Taken together, these facts suggest that a true clinically relevant sensitization to food-contained allergens belonging to the Bet v 1 family could exist in the absence of specific IgE to any birch pollen allergen.

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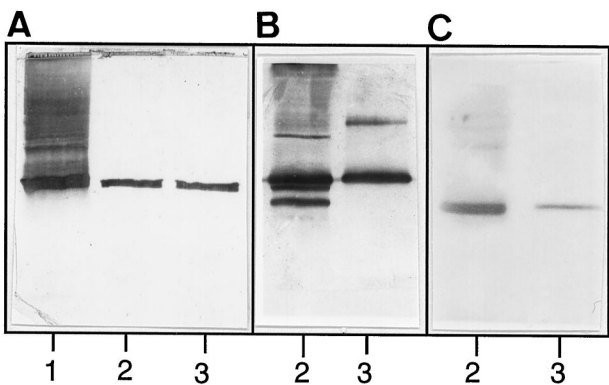


Figure 3. (A) Silver stained SDS-PAGE of the raw carrot extract (lane 1), the Dau c 1 enriched fraction (lane 2) and purified Dau c 1 (lane 3). The last two fractions were tested by blot using the rabbit polyclonal antiserum (B) and a pool of the patients' sera (C).

	1										10									
carrot allergen Dau c 1	G	A	Q	S	H	S	L	E	I	T	S	S	V	V	A	E				
celery Api g 1	G	V	Q	T	H	V	L	E	L	T	S	S	V	S	A	E				
birch Bet v 1	G	V	F	N	Y	E	T	E	T	T	S	V	I	P	A	A				

Figure 4. N-terminal aminoacid sequence of Dau c 1 aligned with Api g 1 (10) and Bet v 1.

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