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Mugwort-fennel-allergy-syndrome associated with sensitization to an allergen homologous to Api g 5

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Summary

Background: *The cross-reactive allergen responsible for the so called "mugwort-celery-spice-syndrome", a pollen-food allergy that occurs in a minority of mugwort pollen-allergic patients, is still undefined.* ***Objective:*** *To identify the allergen responsible for the cross-reactivity between mugwort pollen and plant-derived foods.* ***Methods:*** *The serum from one index patient with both fennel and mugwort pollen allergy was used to identify IgE-reactive allergens by direct ELISA and Immunoblot analysis. Cross-reactivity between mugwort pollen and fennel was checked by cross-inhibition experiments. Fennel and mugwort allergens selected on the basis of IgE reactivity and inhibition tests were excised from SDS-PAGE gels and microsequenced. The amino acid sequences obtained were used to screen the NCBI database using the protein BLAST software.* ***Results:*** *On ELISA inhibition experiments, serum absorption with fennel extract completely inhibited the IgE response to mugwort. On immmunoblot analysis periodate treatment caused the disappearance of all bands of IgE reactivity except one at about 60 kDa. The 60 kDa bands from both mugwort and fennel PAGE-SDS gels revealed the presence of distinct proteins. The N-terminal amino acid sequencing gave the same major amino acid sequence corresponding to an Api g 5-like allergen. The MS/MS spectra were analyzed and a provided evidence of a fennel-specific protein with sequence similarity to phosphoglyceromutase from Apium graveolens.* ***Conclusion:*** *A 60 kDa allergen, highly homologous to Api g 5, was recognized in fennel by patient's IgE. Inhibition experiments showed a high degree of cross-reactivity between this fennel allergen and the homologous mugwort pollen allergen. This allergen might be responsible for the mugwort-celery-spice syndrome.*

Introduction

Patients hypersensitive to mugwort (*Artemisia vulgaris*) pollen may experience allergic reactions following the ingestion of plant-derived foods due to the cross-reactivity between pollen and plant food allergens. Such cross-reactions may occur in at least three different conditions. (a) Sensitization to the plant pan-allergen profilin, which is associated with multiple pollen reactivity and often characterized by oral allergy syndrome following the ingestion of a large array of foods, more typically melon, watermelon, tomato, citrus fruits, and banana (1,2). (b) Cross-reactivity may occur within the Compositae family; anaphylaxis and other types of food allergy following the ingestion of chamomile tea, royal jelly, honey, or sunflower seed have been reported in mugwort allergic patients (3-6). (c) The so-called mugwort-celery-spice syndrome. This sort of pollen-food-allergy syndrome was first reported more than 25 years ago (7,8). The prevalence of the syndrome among mugwort-allergic patients has not been clearly defined but seems rather low out-

side Switzerland (9), suggesting that both a minor mugwort allergen and a peculiar food allergen are involved. Since allergens associated with the syndrome are both heat- and pepsin resistant (10-12) patients may experience systemic reactions following the ingestion of offending foods. Reported offending foods include celery root, anise, fennel, coriander, cumin, pepper, and paprika (8,13,14).
Heiss and co-workers (10) detected a 60 kDa cross-reactive carbohydrate epitope as the allergen responsible for the cross-reactivity between mugwort pollen and celery, an observation that was confirmed also by other researchers (15-17) also in other foods (14). More recently, Api g 5, a 58 kDa celery glyocoprotein allergen, was shown to induce in-vitro basophil histamine release and seemed to have potential to elicit allergic reactions in-vivo (18); however, the allergen concentrations needed to achieve mediator release were relatively high (19), and its biologic activity low (20). Thus, also in view of the little or no clinical relevance of sensitization to cross reactive carbohydrate determinants (21), the identity of the culprit allergen has remained unclear ever since.
In the present study, we report how starting from a few cases of fennel allergy in mugwort-sensitized patients we eventually detected Api g 5-like cross-reacting structures both in fennel and mugwort pollen and provide evidence that this allergen is most probably the cause of the mugwort celery spice syndrome.

Patients and methods

Patients: case reports

Case 1: A 20 year old man with a history of mild grass pollen allergy experienced anaphylaxis a few minutes after the ingestion of a small portion of raw fennel (generalized urticaria, dysphonia, lips angioedema, palm-plantar itch). The allergic reaction subsided at home after the administration of systemic steroids and oral cetirizine. The patient had always tolerated all fruits and vegetables before.
On allergological assessment the patient showed strong skin reactivity to fresh raw fennel (mean wheal diameter 20 mm) and a moderate reactivity to fresh cooked fennel. Skin prick tests with a series of commercial food extracts (ALK-Abellò) scored positive for peanut, hazelnut, and peach, as did a SPT with fresh apple. SPT with a series of commercial extracts of airborne allergens including grass, mugwort, olive, birch, pellitory, cypress and plantain, scored frankly positive for grass and mugwort pollen.
On ImmunoCAP (Thermofisher, Uppsala, Sweden) fennel IgE level was 12,0 KUa/L. On Multiplex ISAC Immunoassay (Thermofisher) the patient reacted to both Art v3 (2.3 ISU), and rPru p 3 (9.4 ISU) (notably, both are lipid transfer proteins), whereas he did not show any reactivity to Art v1.
Although the patient refused to undergo oral challenges with foods involved in the mugwort-celery-spice syndrome other than fennel, fennel, celery, aniseed, caraway, coriander and dill were excluded from his diet . The patient did not experience further episodes of food allergy until some days ago when he experienced a similar reaction following the ingestion of a risotto dish prepared using a vegetal bouillon cube.

Case 2: A 41 year old man with a history of mild perennial rhinitis with seasonal worsening experienced two episodes of oral allergic syndrome and dyspnoea few minutes after ingestion of raw and cooked fennel, respectively. In both occurrences the allergic reaction subsides in two hours without therapy. On the allergological assessment, which was performed three months after the last adverse reaction, the patient showed strong skin reactivity to grass, mugwort, cypress, mites, Alternaria, cat dander, and raw fennel. The level of fennel-specific measured by ImmunoCAP was 2,3 kUA/L, and the ImmunoCAP ISAC microarray confirmed the primary sensitizations to grass, cypress, mites, Alternaria, cat dander. As concerning mugwort, only the LTP (Art v 3) scored positive (0.8 ISU), but no IgE to Art v 1 was recorded. The other LTPs present in the ImmunoCAP ISAC panel scored negative.

In-vitro studies

a) Preparation of extracts

Pollen of short ragweed (*A artemisiifolia*) delivered by Allergon (Engelhom, Sweden) was extracted as a 10 wt/vol suspension in 50 mmol/L phosphate-buffered saline (PBS), pH 7.0, overnight at 4°C in agitation. After centrifuging at 30.000 g for 30 min the supernatant was harvested and dialyzed against the same buffer, passed through a 0.2 μm filter and stored at -20°C.
Fresh fennel or celeriac purchased at a local market was homogenized. The homogenate was mixed with 300 ml of pre-cooled acetone and equilibrated at –20°C overnight. After removal of the supernatant, the precipitates were washed twice with acetone and once with acetone/ether

(1:1, v/v) and then dried carefully . The resulting powder was extracted as previously described (22). Protein concentration of the extracts, measured according to Bradford (2) by BioRad method (BioRad, Milan Italy) were:
Short ragweed pollen: 1.8 mg/ml
Fennel extract: 573 μg/ml
Celery extract: 230 μg/ml

b)Direct ELISA

On direct ELISA, patients' sera were tested against mugwort, fennel, celeriac, and recombinant peach LTP (24), and against bromelain (as a marker of IgE reactivity to CCD; Sigma, Milan, Italy). IgE-reactivity to whole extracts, recombinant LTP, and bromelain of patient's serum and of a control serum (represented by a pool of 5 sera from non-atopic individuals) was measured by ELISA. Two μg of extracts/100 μl coating buffer (15 mmol/L Na_2CO_3 and 35 mmol/L $NaHCO_3$, pH 9.6), or 0.1 μg of recombinant LTP or bromelain /100 μl per well were used in the coating phase for 96-microtitre plates (Maxisorp Nunc, Roskilde, Denmark). After washings with 0.1 M phosphate-buffered saline, pH 7.4 (PBS) and 0.05% Tween 20 (Sigma, Milan, Italy), wells were saturated with 2% bovine serum albumin (BSA) in PBS (diluition buffer) for 2 hours at room temperature. Subsequently, after further washing, 100 μl of either normal or patient's serum diluted 1:3 (in dilution buffer) were added to wells and incubated for 2 hours at room temperature. Wells were washed, and bound specific IgE was detected by adding a peroxidase-conjugated anti-human IgE from goat (1:4000, Biospacific, Emeryville, CA, USA); developing colorimetric reaction was induced by using tetramethyl-benzidine/H_2O_2 as substrate. The enzyme reaction was stopped after 20 minutes by the addition of 1 mol/L HCl. Absorbance values were read at 450 nm by spectrophotometer. In inhibition studies, patient's serum was pre-absorbed for 2 hours at room temperature with different concentrations of various inhibitors (30 μl of sera and 120 μl of dilution buffer containing inhibitors) before the test. IgE levels were expressed as optical density units (OD). Based on the mean + 2SD of IgE levels found in normal control, values less < 300 OD were considered negative.

c) Immunoblot and Immunoblot Inhibition

Patient's IgE reactivity against mugwort and fennel extracts was investigated by immunoblot analysis under reducing conditions. Both extracts were mixed with LDS sample buffer (Nupage Bis-Tris, Novex, Prodotti Gianni, Milan, Italy) and 5 % B-mercaptoethanol. The samples were then denaturated by heating at 100°C for 5 minutes. Electrophoresis of extracts (25 μg/lane) was carried out in a 10% polyacrylamide precast gel (Nupage Bis-Tris, Novex, Prodotti Gianni, Milan, Italy) at 180 mA for 1 h. The resolved proteins were transferred for 1 h onto a nitrocellulose membrane according to Towbin et al. (25). Extracts blotted onto nitrocellulose strips were treated with sodium periodate in order to oxidize glycoprotein oligosaccharides, as previously described (26). The membrane was saturated with 0.1 mol/L tris-buffered saline containing 5% fat-free milk powder and incubated for 16h at 4°C with serum (dilution 1:5 in saturation buffer). After 3 washings, bound specific IgE were detected by peroxidase-conjugated anti-human IgE antibodies from goat (1:5000 in saturation buffer, Biospacific, Emeryville, CA, USA) and using an ECL western blotting kit (Amersham, Milan, Italy) as substrate. In inhibition studies, serum (1:5 in saturation buffer) was pre-absorbed with 300 μg of fennel extract, with 300 μg of an unrelated extract or with 300 μg mugwort extract before mugwort immunoblot.

d) Protein identification

The bands from fennel and mugwort SDS-PAGE gels selected on the basis of the patient serum reactivity and inhibition test were excised, passively eluted on Prosorb (Applied Biosystems, Foster City, CA, U.S.A.) and microsequenced on a Procise 492 protein sequencer (Applied Biosystems, Foster City, CA, USA). The amino acid sequences obtained were used to screen the NCBI database (http://www.ncbi.nlm.nih.gov/) using the protein BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Moreover, the fennel band at about 60 kDa underwent trypsin digestion and LC-MS/MS analysis as already described (27). Data interpretation was achieved by Mascott software (http://www.matrixscience.com/home.html). If necessary, the theoretical amino acid sequences matching the experimental amino acid sequences were tested for signal peptide prediction with the SignalP 4.0 software (http://www.cbs.dtu.dk).

Results

Direct ELISA and ELISA inhibition tests

On direct ELISA patient's 1 serum showed different levels of IgE reactivity to mugwort, fennel, celeriac, and

peach lipid transfer protein (table 1). IgE reactivity to bromelain (a marker of reactivity to cross-reactive carbohydrate determinants, CCDs) was present as well. In view of the unusually strong IgE reactivity against fennel it was decided to perform the following experiments with fennel rather than with celery. On ELISA inhibition experiments, serum absorption with fennel extract completely inhibited the IgE response to mugwort.
Probably due to the lower levels of fennel-specific IgE (see above) patient 2 serum did not react against fennel on direct ELISA and was no longer employed for other in-vitro experiments.

Immunoblot analysis

Mugwort immunoblot analysis showed a complex profile with multiple bands of IgE reactivity at about 20-30, 43, 60 and 94 kDa (figure 1). Fennel immunoblot experiments showed IgE reactivity against two main bands at about 43 and 60 KDa. In order to clarify whether CCDs were involved in IgE reactivity, mugwort extract underwent periodate treatment before being re-analyzed on immunoblot. Such treatment caused the disappearance of all bands of IgE reactivity except the one at about 60 kDa.

In order to evaluate the possible cross-reactivity between mugwort and fennel extracts, mugwort immunoblot inhibition experiments were carried out using fennel extract as inhibitor. The pre-absorption of serum with fennel caused the total disappearance of the 60 kDa band from mugwort immunoblot. Unfortunately, we could not perform immunoblotting inhibition experiments using mugwort extract as inhibitor due to serum shortage. Similarly, due to serum shortage we were not able to study further the 60 kDa allergen by 2D gel analysis, nor we were able to include celery in inhibition experiments.

Protein sequencing and LC MS/MS analysis

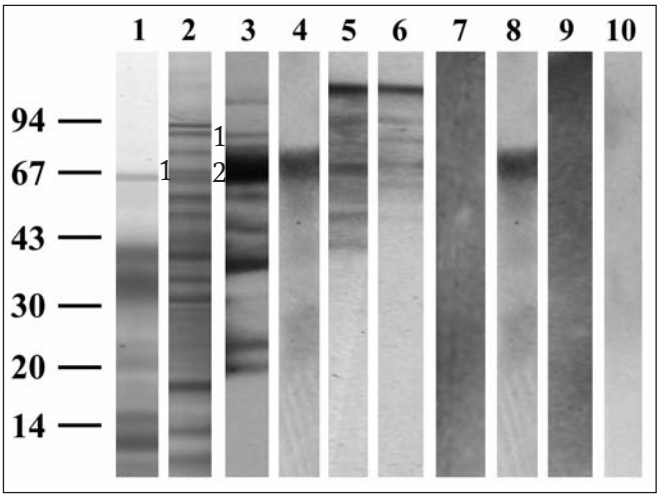
The bands at about 60 kDa from both mugwort and fennel PAGE-SDS gels were amino acid sequenced and revealed the presence of distinct proteins. In the case of mugwort the N-terminal amino acid sequence from band 1 showed two N-terminal sequences, the major one corresponding to AGSKLCEKTSKT, a minor one corresponding to IPNETXVDTS (where X is intended for an undetectable amino acid residue). Bands 1 and 2 from fennel were submitted to N-terminal amino acid sequencing and both gave the same major amino acid sequence

Table 1 *- Direct ELISA and ELISA inhibition results*

EXTRACT	Mugwort	Fennel	Celery	rLTP	Bromelain
Normal control serum	180	148	150	130	436
Patient serum	3484	2822	3665	3905	1855
Patient serum pre-adsorbed with:					
Fennel 5 μg (%inhibition)	401 (88%)				
Fennel 50 μg (% inhibition)	372 (89%)				
Mugwort 0.25 μg		372 (87%)			
Mugwort 2.5 μg		265 (91%)			
Mugwort 25 μg		211 (93%)			
rLTP 0.5 μg	3101 (11%)				
rLTP 5 μg	2815 (19%)				
Mugwort 20 μg	290 (92%)				
Fennel 20 μg		334 (88%)			
BSA 20 μg	3336 (4%)				
BSA 20 μg		2593 (8%)			

IgE levels are expressed as optical density units (OD)

Figure 1 *- SDS-PAGE of mugwort (lane 1), and fennel extract (lane 2). The gels are stained with Comassie colloidal blue (Invitrogen, Milan, Italy). Band 1 from mugwort and bands 1 and 2 from fennel underwent protein identification. Immunoblot analysis. IgE reactivity of patient's serum to mugwort not treated and treated with periodate (lanes 3 and 4, respectively), to fennel extract not treated and treated with periodate (lanes 5 and 6, respectively), to mugwort extract treated with periodate with serum pre-adsorbed with fennel extract, (lane 7), pre-adsorbed with an unrelated extract (Dermatophagoides pteronyssinus) (lane 8), and pre-adsorbed to mugwort extract (lane 9). Control: Lane 10 , IgE reactivity to mugwort of a pool of sera from non-atopic subjects*



corresponding to IPNPAGFNTXLS. The screening of the NCBI database by BLAST software produced the results summarized in Table 2. To try to confirm and better characterize the presence of a Api g 5-like allergen in fennel we decided to "in gel" digest with trypsin the protein-containing bands and to analyse the obtained peptides by LC-MS/MS. The MS/MS spectra were analyzed by Mascot software and a cofactor-independent phosphoglyceromutase from *Apium graveolens* (UniProt entry Q9SDL3, nominal MW 61125) was identified with 7 peptides and a Mascot score of 363 (Table 3). The sequence coverage achieved was 17% (Figure 2).

Discussion

In previous studies, sera from patients with mugwort-celery-spice syndrome were frequently found to react to a 60 kDa protein in celery. Such finding, along with the observation that the same sera often showed IgE reactivity to the major mugwort allergen, Art v 1, at about the same molecular weight (28) led to hypothesize that Art v 1 was responsible for the co-recognition of plant foods in the mugwort-celery-spice syndrome (these data are reviewed in reference 29). However, from a clinical point of view, it seemed odd that only a minority of mugwort-allergic subjects developed a mugwort-celery-spice syndrome if the major pollen allergen is involved in cross-reactivity. In fact, looking at birch pollen allergy as a model, the large majority of subjects sensitized to the major allergen, Bet v 1 score positive on skin tests with plant-derived foods containing Bet v 1-homologous allergens, irrespective of their clinical reactivity (30) which is certainly not the case in the mugwort-celery-spice syndrome. The identification of Api g 5, a celery tuber glycoprotein of 55-58 kDa (31), led to hypothesize a possible homology with the mugwort glycoallergen Art v 60 (10), but such possibility has remained unclear ever since. A 60 kDa allergen possibly cross-reacting with mugwort allergens has been detected in mango as well (32), and also in pepper ad paprika (14). In this study we demonstrate for the first time the existence of a 60 kDa allergen in fennel, that such allergen is recognized by the serum of patients with the mugwort-celery-spice syndrome, and that it is highly homologous to Api g 5. Inhi-

Table 2 *- N-terminal amino acid sequences and their identifications.*

Band number/source	Experimental N-terminal amino acid sequence	Name /entry of matched sequence (UniProt KB)	Amino acid sequence / % of identity
1/ Mugwort	[1]AGSKLCEKTVKT[12] [1]IPNETXVDTS[10]	Art v 1/ Q84ZX5 Artemisinic aldehyde delta-11(13) reductase (Artemisia annua) /ACH61780	[25]AGSKLCEKTSKT[35]/100% [36]IPNEALVEYY[45]/50%
1/ Fennel	[1]IPNPAGFNTXLS[12]	Api g 5 (Apium graveolens) /P81943	[2]PNPSGFVTCLS[12]/73%
2/ Fennel	[1]IPNPAGFNTXLS[12]	Api g 5 (Apium graveolens) /P81943	[2]PNPSGFVTCLS[12]/73%

Figure 2 *- Sequence coverage of the cofactor-independent phosphoglyceromutase from Apium graveolens (Uni Prot entry: Q9SDL3) obtained by Mascot software using data from LC-MS/MS analysis. In bold the identified peptides.*

1 MGSSGVSWKLADHPKLPKGKVVAMIVLDGWGEAHDNQYNCISVAETPTMD
51 SLKNGAPDRWRLVR**AHGTAVGLPTEDDMGNSEVGHNALGAGR**IFAQGAKL
101 CDLALESGK**IYDGEGFNYIK**ESFESGTLHLIGLLSDGGVHSRIDQVLLLV
151 KGASERGAKRIRVHALTDGRDVLDGSSVAFVETLQNYLSELR**EQGIDAQI**
201 **ASGGGR**MYVTMDRYENDWDVVKRGWDAQVLGEAPYKFKSALEAVKTLRAE
251 PKANDQYLPPFVIVDESGKAVGPIVDGDAVVTFNFRADRMVMAAK**AFEYE**
301 **DFDKFDR**VRVPKIRYAGMLQYDGELKLPNHYLVSPPEIDR**TSGEYLTHNG**
351 **VR**TFACSETVKFGHVTFFWNGNRSGYFDSEMEEYVEVPSDSGITFNVQPK
401 MKALEIAEKARDAILSGKFHQVR**VNLPNSDMVGHTGDIAATVVACK**AADE
451 AVKMILDTIEQVGGIYVVTADHGNAEDMVKRNKKGEPALDKDGKIQILTS
501 HTLEPVPIAIGGPGLLPGVRYRKDVPSGGLANVAATVMNLHGFVAPDDYE
551 TTLIEVVDN

bition experiments showed a high degree of cross-reactivity between this fennel allergen and the homologous mugwort pollen allergen. Moreover we clarified that at 60 kDa in mugwort pollen extract there are two different proteins, one corresponding to Art v 1, already cloned and characterized by Himly et al (33), and a second one having the N-terminal amino acid sequence very similar to Api g 5 and to the fennel 60 kDa protein as shown in Table 2. The BLAST search identified a Artemisinic aldehyde delta-11(13) reductase starting from the 36th amino acid residue and with 50% of identity. A signal peptide 35 amino acid long was perfectly predicted by the SignalP 4.0 software. Because the genome of mugwort is not completely known and the N-terminal amino acid sequence of Art v 60 was found as sub-sequence of Art v 1, an identity of 50% is acceptable. Both Api g 5 and Art v 60 are FAD-linked oxidoreductase enzymes but the complete sequence of Api g 5 is still unknown. Concerning the 60 kDa allergen in fennel, the analyses of the tryptic peptides by LC-MS/MS gave the identification of a fennel-specific protein with sequence homology to cofactor-independent phosphoglyceromutase from *Apium graveolens*. The N-terminal sequence of this protein is different from the one that we directly obtained from the N-terminal amino acid sequence analysis. This protein is indicated as "cross-reactive celery allergens" in UniProt Database but its identification as allergen is still unclear. In our opinion it is possible that two proteins are present in the fennel 60 kDa bands, one homologous to Api g5 and the other one similar to the cofactor-independent phosphoglyceromutase. Further investigations are needed to clarify this point.

The mugwort-celery-spice syndrome is very uncommon in Italy (34), and this is the main reason why only two patients were studied here. The only patient showing strong

Table 3 *- List of peptides from Fennel band 1 and 2 identified by LC-MS/MS analysis as cofactor-independent phosphoglyceromutase from Apium graveolens (UniProt entry Q9SDL3) (for sequence coverage see Figure 2)*

Start-end	Observed m/z	MW expected	MW calculated	Amino acid sequence
65-93	916.79	2747.35	2748.25	AHGTAVGLPTEDDMGNSEVGHNALGAGR
110-120	659.63	1317.25	1317.62	IYDGEGFNYIK
193-206	679.62	1357.23	1317.62	EQGIDAQIASGGGR
296-307	791.00	1579.99	1580.68	AFEYEDFDKFDR
341-352	667.19	1332.37	1332.64	AFEYEDFDKFDR
424-446	790.16	2367.46	2368.15	VNLPNSDMVGHTGDIAATVVACK
424-446	795.45	2383.33	2384.15	VNLPNSDMoxVGHTGDIAATVVACK

reactivity on in-vitro assays was sensitized also to food lipid transfer protein as well. Therefore, an allergic reaction to fennel LTP cannot be excluded. Nonetheless, such possibility seems rather unlikely for several reasons: first, the patient never experienced clinical symptoms after eating peaches or other *Rosaceae* that are by far the most frequently offending foods in LTP allergy, up to the point to be regarded as a sort of marker of such condition (35). Second, fennel contains lipid transfer protein but has been very rarely associated with allergic symptoms in patient sensitized to this protein (35).

In conclusion, our study shows the cross-reactivity of a 60 kDa allergen other than Art v 1 present in mugwort pollen, celery, and fennel. This allergen is likely responsible for the "mugwort-celery-spice syndrome". The fact that a minor *Artemisia* pollen allergen is involved in such cross-reactivity explains the relative rarity of this sort of pollen-food allergy syndrome.

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