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Heterogenity of IgE response to walnut and hazelnut in italian allergic patients

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Summary

Background: *The prevalence of IgE reactivity against genuine walnut and hazelnut allergens is poorly defined.* ***Objective:*** *The IgE response to walnut and hazelnut was investigated in Italian patients with primary allergy to these nuts.* ***Methods:*** *Sera from 36 patients allergic to hazelnut and/or walnut, not reactive to PR-10, profilin, and LTP, underwent immunoblot analysis with extracts of both nuts.* ***Results:*** *Most patients had a history of systemic symptoms following the ingestion of the offending food(s). Twelve patients were sensitized to both walnut and hazelnut, and 13 were sensitized to other nuts and seeds (cashew, peanut, sesame, pine nut, almond, Brazil nut, and pistachio). On walnut immunoblot, the 7 sera which scored positive showed much variability in their IgE profile. Two reacted uniquely at 10 kDa, and the others at 35 , 40, 45, 50, 67, and > 67 kDa. The profiles obtained under reducing and non-reducing conditions showed several differences. The 7 sera positive on hazelnut immunoblot under reducing conditions recognized sera at 10 kDa and at <10 kDa (n=1), 20 kDa (n=4), at about 22, 24, 30, 40, 43, 58, 60, and 90 kDa, and higher m.w. in other cases. Under non-reducing conditions IgE reactivity at 20, 28, 35, 40, 45, 60, 90, and 100 kDa, was detected. Only two sera scored positive under both conditions and showed an IgE profile that partly changed from one assay to another.* ***Conclusion:*** *The current list of walnut and hazelnut allergens is far from being complete. Both reducing and non-reducing conditions are needed to detect IgE reactivity in individual patients*

Introduction

Walnut and hazelnut are by far the most commonly consumed tree nuts worldwide. Food allergy to these plant-derived foods has been rather frequently reported. Most cases of walnut and hazelnut allergy occur in patients primarily sensitized to birch pollen or sensitized to the pollen pan-allergen profilin due to the cross-reactivity between Bet v 1, the major birch pollen allergen, and pollen profilin and the homologous allergens in the two fruits (1,2). In the Mediterranean area many cases of walnut and hazelnut allergy occur in patients primarily sensitized to the peach due to the cross-reactivity between the lipid transfer proteins from different food sources (3). Primary sensitization and allergy to tree nuts is much less common, at least in Italy (4,5), although of great clinical relevance due to the extreme severity of clinical consequences in allergic subjects. A large number of allergens have been detected in walnut and hazelnut so far (table 1; Ref 6-17). The prevalence of IgE reactivity against these allergens among allergic subjects is ill defined. In the present study we aimed to investigate the IgE response to walnut and hazelnut in Italian patients with primary allergy to these nuts.

Patients and methods

Patients

The study was carried out on patients diagnosed as having hazelnut and/or walnut allergy at 33 Italian allergy departments from January 1st to December 31st, 2011. Diagnosis had to be based on a clear-cut clinical history of oral allergy syndrome, asthma, urticaria/angioedema, and/or anaphylaxis following the ingestion of walnut and/or hazelnut associated with an unequivocally positive SPT with fresh fruit and/or commercial extract. Since the objective of this study was to investigate the IgE reactivity in genuine walnut and/or hazelnut allergic patients, patients sensitized to cross-reacting plant-food allergens, namely PR-10, profilin, and LTP were excluded (see below). Included patients were thoroughly interviewed to detect their clinical reactivity to foods other than walnut and/or hazelnut, particularly sesame, almond, peanut, pine nut, Brazil nut, and sunflower seed. The study was carried out as a part of the routine clinical activity of all participating centers, hence no formal approval by an Ethical Committee was required. All study patients gave an informed consent to the serological analyses.

Skin tests

Walnut and hazelnut hypersensitivity was detected by SPT with fresh material using the prick-prick technique and with commercial extracts (ALK-Abellò, Madrid, Spain). Hypersensitivity to PR-10, profilin, and LTP was excluded on the basis of negative SPT with a commercial birch pollen extract, with a commercial extract of date palm pollen enriched in profilin (Pho d 2, 50 μg protein/ml; ALK-Abellò, Madrid, Spain), and with a commercial peach allergen (30 μg LTP/ml; ALK-Abellò), respectively. In some centers sensitization to these allergens was ruled out by in-vitro testing, showing the absence of IgE to Bet v 1, Bet v 2, and Pru p 3/Cor a 8 by ImmunoCAP. All skin tests were carried out and read following

Table 1 - Current list of allergen proteins detected in hazelnut and walnut

	Hazelnut	Walnut
PR-10	Cor a 1 (18 kDa) [6]	
Profilin	Cor a 2 (14 kDa) [7]	Jug r 5 (14 kDa) [13]
2-S Albumin	Cor a 14 (17 kDa) [8]	Jug r 1 (15 kDa) [14]
Vicilin (7-S globulin)	Cor a 11 (48 kDa) [9]	Jug r 2 (48 kDa) [15]
Legumin (11-S Globulin)	Cor a 9 (40 kDa) [10]	Jug r 4 (58 kDa) [16]
Lipid transfer protein	Cor a 8 (10 kDa) [11]	Jug r 3 (10 kDa) [17]
Oleosin	Cor a 12 (17 kDa)[12] Cor a 13 (14-16 kDa)	
Thaumatin-like protein	Cor a TLP (25 kDa) [13]	

established criteria. Only wheals showing a mean diameter exceeding 3 mm at 15 min were considered as a positive response. SPT with histamine 10 mg/ml and saline were used as positive and negative controls, respectively (18)

In-vitro studies

Eight grams of walnut or hazelnut, defatted with hexane, were extracted for 1 hour in 100 ml of 0.9 M NaCl, at 4 °C under stirring. After centrifuging, the supernatant was harvested and dialyzed against the same buffer. The protein content, measured by Bradford's method (19), was 1.8 mg/ml for walnut and 3.2 mg/ml for hazelnut. The electrophoresis of walnut and hazelnut extract (25 μg per lane) was carried out in a 10% polyacrilamide precast Nupage Bis-Tris gel according to manufacturer instructions (Invitrogen, Milan, Italy) at 180 mA for 1 h under both reducing and non-reducing conditions. The resolved proteins were transferred onto a nitrocellulose membrane (Protran BA 85, Schleicher and Schuell, Milan, Italy) according to Towbin (20). The membrane was saturated in TBS buffer containing 5% defatted dry milk (saturating buffer) and incubated with patient's serum or normal serum diluted 1:5 in saturating buffer. Bound specific-IgE were detected by adding peroxidase-conjugated anti-human IgE from goat (diluted 1:8000, Biospacific, Emeryville, CA, USA) and ECL western blotting kit (Amersham, Milan, Italy) as substrate.

Results

Patients

Thirty-six patients (aged 4-53 years [mean age 21,4 years; median 19 years]; M/F ratio 10/26) diagnosed at 14 allergy centers fulfilled the admission criteria and were included in the study (table 2). Twelve patients were sensitized to both walnut and hazelnut on SPT; 6 of them had a history of allergy to both foods, and 6 had a history of allergic reactions only to one of them (walnut in 4 cases; hazelnut in 2). Twenty one patients were allergic and sensitized only to one of the two foods (walnut in 11 cases; hazelnut in 10). In two cases, 1 allergic to walnut and 1 to hazelnut, sensitization to the other food was not ascertained. Sensitization to other nuts and seeds was detected in 13 cases (cashew 3, peanut, sesame, pine nut and almond 2 each, Brazil nut and pistachio 1), and in 7 cases this was associated to clinical allergy (Pine nut 3, Brazil nut 2, almond and sesame 1) (Table 2).
Most patients had a clinical history of systemic symptoms following the ingestion of the offending food(s); in some cases these were extremely severe. Eleven patients reported only local oral or gastrointestinal symptoms following the ingestion of walnut and/or hazelnut.

Immunoblot analysis

a) Walnut
Of 22 walnut-allergic patients only 7 scored positive on immunoblot analysis under reducing conditions (Figure 1). IgE reactivity showed much variability. Serum from two patients (no. 10 and 24) showed IgE reactivity uniquely at about 10 kDa. The remaining 5 patients reacted against proteins at about 35 kDa (n=1), 40 kDa (n=1), 45 kDa (n=4), 50 kDa (n=2), 67 kDa (n=5), > 67 (n=3). Sera from 4 patients scored positive also on immunoblot carried out under non-reducing conditions (figure 2). The immunoblot profiles obtained under the different conditions showed several differences (figures 1 and 2)

b) Bazelnut
Of 19 patients allergic to Hazelnut, 7 scored positive on immunoblot under reducing conditions (figure 3). Allergens at 10 kDa and at <10 kDa were recognized by 1 serum. Four/7 reacted against a 20 kDa allergen, which was the only allergen recognized in 3 cases. Further IgE reactivity was observed at about 22 kDa (n=1), 24 kDa (n=1), 30 kDa (n=1), 40 kDa, (n=1), 43 kDa (n=1), 58 kDa (n=1), 60 kDa (n=1), 90 kDa and more (n=1). One patient showed IgE reactivity to a large number of allergens at different molecular weights. One patient reacted only against high m.w. allergens (60 kDa and more).
Sera from 6 patients showed some IgE reactivity to hazelnut on immunoblot carried out under non-reducing conditions. Of these, only 2 coincided with those positive on immunoblot performed under reducing conditions (figure 4). IgE reactivity at 20 kDa (n=1), 28 kDa (n=1), 35 kDa (n=1), 40 kDa (n=3), 45 kDa (n=2), 60 kDa (n=2), 90 kDa (n=1), and 100 kDa (n=1) was detected. The two sera scoring positive under both reducing and non-reducing conditions showed an IgE profile that changed, at least in part, from one assay to another. For instance, one serum (no. 40) that showed IgE reactivity only at 20 kDa under reducing conditions (figure 3) reacted uniquely at about 40 kDa under non-reducing conditions (figure 4).

Table 2 - Clinical features of study patients.

Patient	Age	Sex	Offending food & clinical symptoms	Other nuts/seeds positive on SPT
1	24	F	Walnut (U,G); Hazelnut (U,G)	Cashew, Almond
6	9	M	Hazelnut (U)	Walnut, Peanut, Cashew, Brazil nut
10	12	F	Walnut (U,A); Hazelnut (U,A)	Sesame
11	4	M	Walnut (U); Hazelnut (U)	Sesame
12	8	F	Hazelnut (U,A)	Walnut
15	22	F	Walnut (O)	-
17	13	F	Hazelnut (A)	-
18	31	F	Walnut (O), Brazil nut (O)	- (Brazil nut ND)
19	7	F	Hazelnut (O)	-
21	7	F	Hazelnut (O)	-
23	10	F	Walnut (U,A),	Hazelnut, Cashew, Almond, Pistachio
24	7	F	Walnut (X)	-
25	13	F	Walnut (X)	-
29	12	M	Walnut (G,A)	-
33	20	F	Hazelnut (O)	-
35	34	M	Walnut (O)	-
38	16	F	Walnut (O)	-
40	8	F	Hazelnut (X)	-
41	75	F	Walnut (U)	-
43	22	M	Hazelnut (X)	-
51	53	M	Hazelnut (A, O)	-
55	21	M	Hazelnut (U)	-
56	15	F	Hazelnut (U,X)	-
61	47	F	Walnut (X). Hazelnut (U)	-
62	21	F	Walnut (U)	-
63	20	F	Walnut (O), Pine nut (O)	Pine nut
65	53	F	Walnut (X), Hazelnut (O). Almond (O), Pine nut (O), Brazil nut (O), Sesame (O	Almond, Sesame, Pine nut, Brazil nut
81	42	F	Walnut (O)	Hazelnut
82	19	F	Walnut (U)	Hazelnut
83	15	M	Hazelnut (O), Almond (O)	Almond
84	26	F	Walnut (A,G,O)	-
92	9	F	Walnut (U,A,G), Hazelnut (U,A,G), Pine Nut (U,A,G)	- (Pine nut ND)
93	8	M	Walnut (U,G,A)	Hazelnut, Peanut
103	23	F	Hazelnut (O)	ND
106	25	F	Walnut (U)	ND

X: Anaphylaxis; U: Urticaria/angioedema; O: Oral allergy syndrome; G: gastrointestinal symptoms; A: Asthma. ND: the skin tests for these foods were not done.

Figure 1 - Immunoblot analysis with walnut extract performed under reducing conditions. Numbers at the base of each lane represent the number of serum. NS: Normal control serum

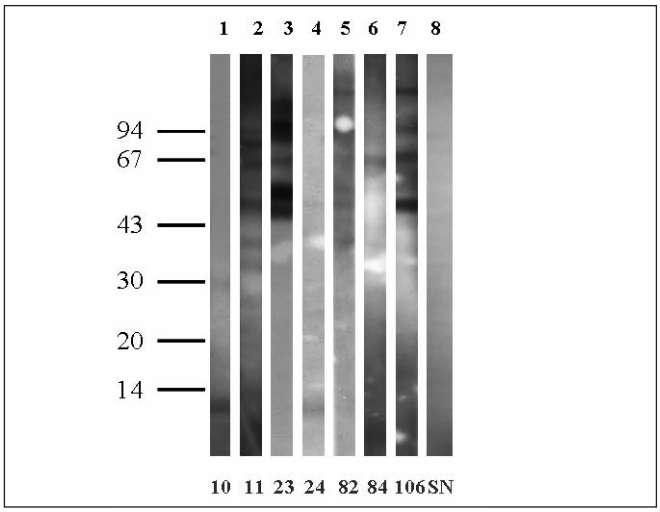
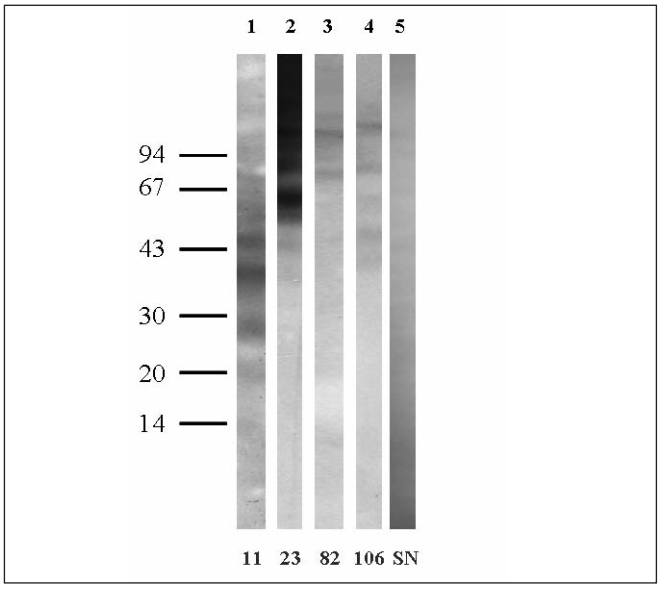


Figure 2 - Immunoblot analysis with walnut extract performed under non-reducing conditions.



c) Walnut & Hazelnut

Sera from only 2/6 patients (sera no. 10 and 11) with a history of allergy to both walnut and hazelnut showed IgE reactivity to both fruits on immunoblot. Interestingly, both sera showed sesame hypersensitivity as well. Comparing the immunoblot profiles, patient #11 showed IgE reactivity against a 30 kDa allergen in both walnut and hazelnut, and further reactivity to 40 kDa and 48 kDa proteins in walnut.

Figure 3 - Immunoblot analysis with hazelnut extract performed under reducing conditions

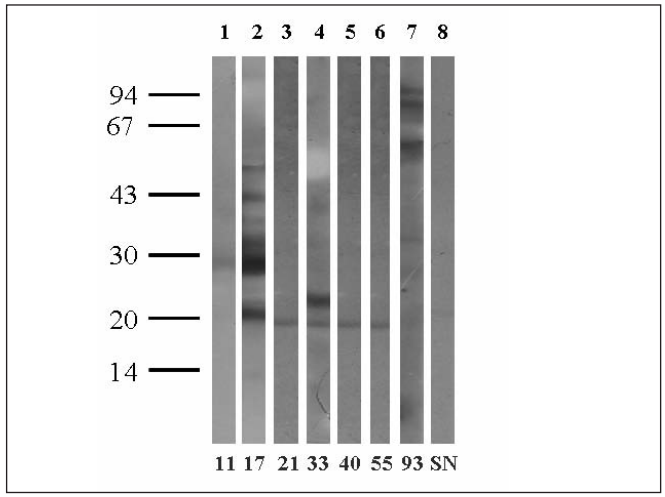
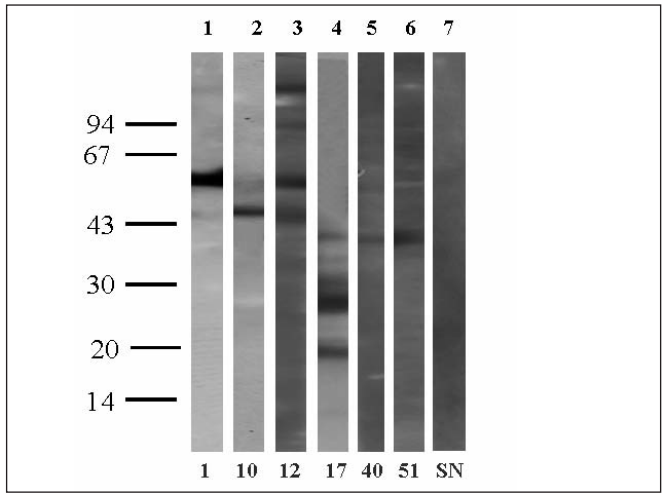


Figure 4 - Immunoblot analysis with hazelnut extract performed under non-reducing conditions.



In contrast, patient #10 showed completely different profiles against the two fruits (figures 1 and 3). In general co-sensitization to nuts other than walnut and hazelnut or to seeds was not associated with a specific immunoblot profile. Finally, a positive immunoblot assay was not associated with a history of more severe allergic reaction.

Discussion

This study aimed to investigate the IgE reactivity of patients with a genuine walnut and hazelnut allergy. To this

end, patients sensitized to birch pollen, profilin and non-specific lipid transfer protein, which represent by far the majority of subjects sensitized to tree nuts in Italy, were excluded. In fact, in a previous study on more than 1000 adult patients with food allergy (4) only 27 and 38 were primarily allergic to walnut and hazelnut, respectively, compared to 43 and 42 patients sensitized to LTP and more than 300 subjects with type 2 allergy. One interesting finding was that primary walnut and hazelnut allergy was observed mainly in young patients, although most participating centers cared mostly adults. This fact suggests that, at least in Italy, sensitization to genuine tree nut allergens occurs at a rather young age in most cases. A significant proportion of patients included in this study had sensitization, and often a clinical history of allergy as well, to both walnut and hazelnut. Walnut and hazelnut are taxonomically unrelated, belonging to the families of Juglandaceae and Corylaceae, respectively. However, cross-reactivity between different nuts is found in about one third of patients (21) albeit this occurs more frequently between nuts from the same taxonomic family (22-25). Rather surprisingly, the few patients sensitized to both walnut and hazelnut who scored positive on immunolblot did not show any specific IgE profile. Our findings confirmed that in a minority of patients allergic to walnut and/or hazelnut, clinical sensitization and/or allergy to other nuts/seeds may occur. We did not investigate whether such cases of co-sensitization or multiple nut allergy were in fact cases of co-recognition of homologous cross-reacting allergens, since this was not the objective of this study. Nonetheless a combination of walnut/hazelnut/Brazil nut hypersensitivity has been described before (26).

Possibly due to the low concentration of specific IgE in sera, immunoblot analysis was not able to clarify the IgE profile in a large proportion of our allergic patients; this is not particularly surprising, in view of the low sensitivity of this in-vitro technique. Patients' sera who scored positive in-vitro showed a large variability in their IgE response to walnut and hazelnut. Interestingly, among walnut allergic patients, 4 sera reacted at a molecular weight corresponding to the mass of Jug r 2, the walnut vicilin (48 kDa). All other proteins recognized by patients' sera showed molecular weights other than those of the major walnut allergens described so far (Jug r 1, 15 kDa and Jug r 4, 58 kDa). Among sera from hazelnut-allergic patients, altogether 5 reacted against a protein whose molecular weight corresponded to that of 2-S albumin (Cor a 14; 17 kDa) under either reducing and non-reducing conditions. Three sera reacted at about 40 kDa, and 3 sera at about 48 kDa, corresponding to the molecular weight of hazelnut legumin and vicilin, respectively. All other allergen proteins recognized by the sera from allergic patients showed different molecular weights, not corresponding to those of allergens described so far (13). The differences found using reducing or non-reducing conditions in immunoblot analysis might reflect the different physical/chemical features of several allergens, and suggest that in order to detect IgE reactivity to some proteins carrying out in-vitro testing in both conditions may be needed. Altogether, this study confirms that primary tree nut allergy is a rather rare phenomenon, that most cases of allergy to these fruits are caused by sensitization to panallergens (4, 27), and that that the current list of walnut and hazelnut allergens is far from being complete. It further shows that skin tests as well as in-vitro tests using whole food extracts rather than recombinant allergens still remain an invaluable tool to diagnose this kind of food allergies.

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