

The *Helix aspersa* (Brown Garden Snail) Allergen Repertoire

Luís Miguel Lourenço Martins^a Gabriel Peltre^c Carlos José Fialho da Costa Faro^b
Euclides Manuel Vieira Pires^b Filipe Fernando da Cruz Inácio^a

^aUniversity of Évora, Évora, and S. Bernardo Hospital, Setúbal, and ^bUniversity of Coimbra, Coimbra, Portugal; ^cÉcole Supérieure de Physique et Chimie Industrielles, Paris, France

Key Words

Allergens · Cross-reactivity · *Helix aspersa* · Mites · *Otala lactea* · Snails · *Theba pisana*

Abstract

Background: Ingestion of snails can induce strong asthmatic or anaphylactic responses, mainly in house-dust-mite-sensitized patients. The aim of this study was to identify the *Helix aspersa* (Hel a), *Theba pisana* (The p) and *Otala lactea* (Ota l) allergens and the extent of their cross-reactivity with the *Dermatophagoides pteronyssinus* (Der p) mite. **Patients and Methods:** In 60 atopic patients, skin prick tests (SPT) to snail and *D. pteronyssinus*, total and specific IgE, specific IgE immunoblots, RAST and immunoblot inhibition assays were performed. **Results:** Mean total IgE was >1,000 kU/l. Mean specific IgE (class 6 for Der p and class 2 for Hel a) SPT were positive in 44 patients for snail and in 56 for mite. Isoelectric focusing (IEF) and SDS-PAGE followed by immunoblotting of *H. aspersa* extract enabled the identification of 27 and 20 allergens, respectively. Myosin heavy chains from snails (molecular weight >208 kDa) disclosed

two major allergens. Hel a and Der p RAST were strongly inhibited by their homologous extracts, with Hel a RAST being inhibited by the Der p extract to a much greater extent (72.6%) than the inverse (5.6%). A complete inhibition of the immunoblots by their homologous extract was obtained. However, Hel a extract did not inhibit Der p IEF separated recognition. On the other hand, mite extract extensively inhibited snail immunoblots from both IEF and SDS-PAGE separations. Immune detection on chicken, pig, rabbit, cow and horse myosins did not reveal any IgE cross recognition with snail. **Conclusions:** In most cases of snail allergy, mite appeared to be the sensitizing agent. Nevertheless, snails may also be able to induce sensitization by themselves. This hypothesis is supported by the finding of specific IgE to Hel a in 2 patients who did not show specific IgE to Der p, and one of them was suffering from asthma after snail ingestion.

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Introduction

Snails are a very popular delicacy in European countries and probably an important source of nutrients, since they have a remarkable protein and different salt concentration and low muscle fat. However, some people develop allergic reactions after the ingestion of snails, mostly consisting of severe [1] episodes of asthma.

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Correspondence to: Dr. Luís Miguel Lourenço Martins
Departamento de Sanidade
Universidade de Évora, Núcleo da Mitra
PT–7000 Évora (Portugal)
Tel. +351 21 2060177, Fax +351 266 760824, E-Mail lmlm@uevora.pt

Allergy to snails might be important in countries like France, Italy, Portugal or Spain, as it is known that the prevalence of allergic reactions to a specific food is mainly dependent on the population's eating habits. Allergy to fish in Scandinavia [2] and to soybean in Japan [3] are examples of this.

Snail hypersensitivity was firstly reported by Palma Carlos et al. [4] in 1985, but hypersensitivity to various species belonging to different classes of *Mollusca phylum* has been reported for several times [5–14]. Other studies have already indicated a connection between snails and inhalant allergens, such as house dust mites [15–27].

Typically, allergen extracts contain a great number of components from which a small portion binds to detectable amounts of serum IgE, the allergens. Food allergens are in general glycoproteins with a molecular weight (MW) of 15–50 kDa. Their immunogenicity depends on the MW facilitating digestive absorption, as well as on the number of epitopes on the antigen molecule [28]. Generally, people developing immediate reactions to food are atopic patients [29] who suffer from respiratory allergic disease [30]. The population we have studied fitted well into this clinical pattern.

The aim of this study was to determine the *Helix aspersa* (Hel a), *Theba pisana* (The p) and *Otala lactea* (Ota l) allergen repertoires and to extend the knowledge on snail/mite cross-reaction.

Patients and Methods

Patient Selection and Characterization

From an atopic population, 60 patients with a specific IgE to *H. aspersa* of class 2 or higher (Pharmacia Cap System RAST FEIA) were selected (38 males and 22 females, 32 >15 years and 28 <15 years). Mean age was 14 years. Eighteen patients suffered from asthma, 36 from asthma, rhinitis and conjunctivitis, 3 from rhinitis and conjunctivitis, 2 from atopic eczema and 1 from irritative cough. Six individuals complained of asthma after snail ingestion.

Specific IgE to *D. pteronyssinus* and total IgE were also determined (Pharmacia Cap System IgE FEIA). Skin prick tests (SPT) were done with snail, mite and grass pollen extracts from Stallergenes (Antony, France).

Allergen Extract Preparation

First, snails were cleaned from natural food residues and possible respiratory-tract-contaminating mites. Afterwards, they were submitted to two freeze/thaw cycles to promote tissue disruption and antigen exposure. Then they were shelled, macerated to a uniform mass and submitted to extraction by orbital stirring at a 1:3 dilution in double-distilled water (for isoelectric focusing, IEF) and in PBS (for SDS-PAGE and RAST inhibitions) for 2 h at 4°C. The extract was centrifuged at 25,000 g for 20 min, and supernatant protein concentration was measured according to the Warburg Christian Meth-

od [31] in a spectrophotometer (Perkin Elmer, Norwalk, Conn., USA) and kept in lyophilized aliquots until used. *D. pteronyssinus* extract was obtained by an identical protocol using lyophilised mites (Pharmacia/Allergon, Angelholm, Sweden).

Extracts from chicken, pig, rabbit, cow and horse skeletal muscle were prepared to detect possible cross-sensitization between these commonly eaten muscle proteins and snail. The extraction was performed in a tube rotator, for 15 min at 4°C in 0.6 M KCl to enrich myosin chains. Extracts were centrifuged at 2,000 g, and supernatants were diluted with 14 volumes of cold double-distilled water to promote myosin precipitation and centrifuged at 4,000 g [32]. Myosin obtained from each species was also digested with pepsin at 37°C, pH 2, for 1 h [33], for in vitro digestive system mimesis.

Protein Separation IEF

H. aspersa extract (protein concentration: 6.5 mg/ml) was subjected to pH 3–10 IEF in 4% polyacrylamide gel (acrylamide and ampholytes from Pharmacia Biotech, Uppsala, Sweden) and to pH 2.5–6.5 IEF in 0.7% agarose (FMC, Rockland, Maine, USA)/0.9% sorbitol (Merck, Darmstadt, Germany) gel for immunoblot detections. Separated proteins were then blotted onto 0.2-µm-pore nitrocellulose membranes [34] (Bio-Rad, Hercules, Calif., USA). *H. aspersa* extract was also subjected to pH 2.5–6.5 IEF in 4% polyacrylamide gel, to produce the one-dimensional gel strips for the two-dimensional gel electrophoresis. *D. pteronyssinus* extract containing 9.6 mg/ml of double-distilled water was subjected to pH 4–6.5 IEF in 4% polyacrylamide gel and blotted onto 0.2-µm-pore nitrocellulose membranes.

SDS-PAGE

Extracts from *D. pteronyssinus*, *H. aspersa*, *O. lactea* and *T. pisana* (protein concentration: 8 mg/ml) were treated with SDS under non-reducing conditions and submitted to 8–18% PAGE gradient. Separated proteins were blotted onto 0.2-µm-pore nitrocellulose and onto methanol-activated PVDF membranes (Bio-Rad) in a Nova Blot semi-dry system (Pharmacia Biotech) [35].

Two-dimensional separation of *H. aspersa* was performed by 8–18% SDS-PAGE of the one-dimensional (IEF) separated extract. Separated proteins were blotted onto 0.2-µm-pore methanol-activated PVDF membranes.

Non-digested and pepsin-digested myosin extracts from chicken, pork, rabbit, beef and horse meat were subjected to 8–18% SDS-PAGE separation and then blotted onto methanol-activated PVDF membranes.

Antigen Detection – Immunoblotting

Blotted membranes were cut in 3-mm-wide strips, immersed for 1 h in blocking buffer (PBS, 0.1% Tween 20 and 1% BSA) at room temperature, and each strip was incubated overnight with individual patient sera diluted 1:10 in 1 ml blocking buffer. After incubation, strips were washed four times (5 min) in PBS, 0.1% Tween 20. For specific IgE detection, strips were incubated for 2 h with ¹²⁵I anti-human-IgE (Pharmacia Diagnostics, Uppsala, Sweden) diluted to 20,000 cpm/strip in blocking buffer [36] and washed four times in PBS/0.1% Tween 20. Detection was performed by autoradiography in a cassette with an intensifier screen for 5–14 days at –70°C. Two films (Kodak X-OMAT AR film, Rochester, N.Y., USA) were applied to each cassette, allowing sharper bands by reducing the effect of dispersed radiation. IgE detection was also performed by chromogenic reaction with nitroblue tetrazolium/5-bromo-4-chloro-

Table 1. Clinical and IgE patterns of 6 snail-reactive patients

Patient No.	Total IgE kU/l	Specific IgE kU/l	SPT	Symptoms
5	633	Hel a = 5.22 Der p < 0.35 Dac g = 96.2	Hel a = ++ Der p = ++ Dac g = ++++	polinosis with seasonal asthma, rhinitis and conjunctivitis
13	360	Hel a = 11.5 Der p = 93.8 Dac g < 0.35	Hel a = +++ Der p = ++++ Dac g = -	asthma, rhinitis and conjunctivitis
31	1,907	Hel a = 5.88 Der p = 10.3 Dac g = 885	Hel a = - Der p = ++++ Dac g = ++++	polinosis with seasonal asthma
38	958	Hel a = 2.03 Der p = 86 Dac g < 0.35	Hel a = +++ Der p = ++++ Dac g = ++++	asthma, rhinitis and conjunctivitis
47	600	Hel a = 1.35 Der p = 204 Dac g = 7.19	Hel a = + Der p = ++++ Dac g = ++++	asthma, rhinitis and conjunctivitis
53	587	Hel a = 1.11 Der p = 294 Dac g < 0.35	Hel a = ++ Der p = +++ Dac g = -	asthma, rhinitis and conjunctivitis

3-indolyl phosphate (Sigma, St. Louis, Mo., USA), using alkaline-phosphatase-labelled mouse monoclonal anti-human-IgE (Sigma, 1:1,000) in blocking buffer. IgG4 detection was performed with a pool of sera, followed by chromogenic reaction using biotin-labelled mouse monoclonal anti-human-IgG4 at 1:15,000 and ExtrAvidin-alkaline phosphatase (Sigma). Two controls were applied, one without serum and another with neither serum nor secondary antibody.

Two-dimensional immunoblots were performed for *H. aspersa* by incubating the blotted membrane with a pool of 15 patient sera, diluted 1:10 in 15 ml blocking buffer. Specific IgE was detected with ¹²⁵I anti-human-IgE in autoradiography. IgE detection on blot strips from chicken, pork, rabbit, beef and horse meat myosin was also performed with patient sera (including snail-reactive sera), followed by autoradiographic detection. Control sera from individuals without specific IgE to *H. aspersa* with (6 sera) and without specific IgE to *D. pteronyssinus* (10 sera) were also tested.

Immunoblot Inhibition

Prior to use, extracts were inactivated by heating in a water bath to 60°C for 1 h, to produce enzyme denaturation. Inhibition reactions were performed by 2-hour pre-incubation (under gentle shaking at room temperature) of different heat-inactivated extracts (protein concentration: 20 mg/ml) in a pool of 16 patient sera with specific IgE to Hel a and Der p and a complex spectrotpe in snail immunoblots. Inhibitions were performed with 100 µl of the serum pool and 100 µl of each inhibitor extract.

The following immunoblot inhibitions were performed:

Blot strips	inhibited by	Extracts
Hel a IEF pH 2.5–6.5		Hel a; Der p
Hel a SDS-PAGE 8–18%		Hel a; Der p; The p; Ota I
Der p IEF pH 4–6.5		Der p; Hel a

Immune detection was revealed by autoradiography.

RAST Inhibition

RAST inhibitions were performed by 2-hour pre-incubation of 20 µl of sera with 30 µl of four progressively increasing dilutions (1:1, 1:10, 1:100 and 1:1,000) of heat-inactivated inhibitor extract in PBS-0.03% human albumin (Sigma) under gentle stirring according to the Pharmacia Cap System RAST FEIA protocol. The 0% inhibition was determined by incubating serum samples of 20 µl with 30 µl PBS-0.03% human serum albumin instead of the extract dilution.

The following RAST inhibitions were performed:

Immunocaps	inhibited by	Extracts
Hel a		Hel a (24.5 mg/ml); Der p (20 mg/ml)
Der p		Hel a (24.5 mg/ml); Der p (20 mg/ml)
Pan b		Der p (20 mg/ml)

All the assays were repeated, and the values obtained fell within the Pharmacia Cap System level of acceptance.

Results

Specific IgE to *D. pteronyssinus* (mean value = 229.9 kU/l) was found in 56 patients and specific IgE to *Dactylis glomerata* (Dac g – mean value 81.4 kU/l) in 43 patients. Mean total IgE was 1,295.4 kU/l. SPT were positive in 44 patients for snail, in 56 for mite and in 43 for grass pollen. The 4 non-responders to mite were grass-pollen-allergic patients. The 6 patients complaining of asthma upon ingestion of snails presented different clinical and analytical profiles (table 1).

Table 2. Allergens to *D. pteronyssinus* and *H. aspersa* recognized by IEF

Der p	Hel a	
pI	pI	Recognition (n = 60 patients)
–	3.3	6
–	3.4	43
–	3.6	50
–	3.75	46
–	3.9	1
–	4	11
–	4.2	20
4.25	4.25	8
	4.3	16
4.4	4.4	26
4.45; 4.5;	4.5	15
4.55		
4.65; 4.7	4.7	8
4.95; 5.05	5	9
5.15	5.1	9
5.2	5.2	15
	5.3	28
5.4		
5.5; 5.55	5.5	26
5.6		
5.7; 5.75	5.7	14
5.8		
5.9	5.9	23
6		
6.1	6.1	24
	6.3	36
–	6.4	20
–	6.6	1
–	7	1
–	7.05	1
–	7.4	2
–	7.6	1

Allergen Identification

From IEF separation, 27 allergens were identified from *H. aspersa*, 4 of them with major allergen recognized: pI 3.4; 3.6; 3.8 and 6.3 (fig. 1, table 2). In patients 9, 11, 15, 17 and 24, *H. aspersa* was not detected by IEF (fig. 1). None of them revealed clinical reactions after ingesting snails, but all presented positive SPT for snail. From *D. pteronyssinus* IEF, 21 bands were identified in patients with specific IgE (fig. 2c, tab. 2). The IgG4 spectrotypes followed closely the IgE pattern. From SDS-PAGE separation, 20 allergens to *H. aspersa* were detected. Two of them (MW >208 kDa) showed major recognition, one having been recognized 13 times and the other 18 times in a total of 21 patients (fig. 3, table 3). In all the 6 snail-reactive patients, at least one of these major allergens was



Fig. 1. IgE immunoblots with *H. aspersa* extract submitted to pH 2.5–6.5 IEF in agarose gel and blotted onto nitrocellulose membranes. Thirty-two patients (lanes 1–32) are shown. B = Controls without serum. IgE binding to nitrocellulose-blotted proteins was detected by ^{125}I anti-human IgE in autoradiography. Allergens with major recognition are indicated (arrows).

found (fig. 4). The 208-kDa and the >208-kDa bands have been also recognized in 3 patients with specific IgE to *P. borealis* and to *D. pteronyssinus*, but not to *H. aspersa*. In *T. pisana* and *O. lactea* spectrotypes, 16 and 14 allergens were identified by the 6 snail-reactive patients, respectively (fig. 4).

In patients 22, 40 and 48, *H. aspersa* was not detected by SDS-PAGE (fig. 3), and in 2 of them, patients 40 and 48, specific IgE for *D. pteronyssinus* was also absent. Control sera without specific IgE for *H. aspersa*, with (6 sera) or without specific IgE for *D. pteronyssinus* (10 sera), did not show IgE recognition in blots from the three snail species.

The 6 snail-reactive patients (5, 13, 31, 38, 47 and 53) showed a very close pattern of IgE recognition for the three snail species, with a permanent recognition of the bands >208 kDa (fig. 4). In 2 of them, patients 5 and 31, the spectrotypes for mites was absent (fig. 3). Patient 5 recognized several allergens to *H. aspersa* by IEF (fig. 1) and SDS-PAGE (fig. 4) without specific IgE to *D. pteronyssinus*. Patient 12 also demonstrated several allergens to *H. aspersa* but not to *D. pteronyssinus* (fig. 1, 3).

Immune recognition over chicken, pork, rabbit, beef and horse meat myosin did not present IgE recognition.

Two-Dimensional Separation

Two-dimensional immunoblots of *H. aspersa* disclosed several allergen spots from pI 3.05 to 5.9 and from 35 to >208 kDa (fig. 5).

Fig. 2. IgE immunoblots with *H. aspersa* extract submitted to 8–18% PAGE gradient and blotted onto activated PVDF membranes (**a**), subjected to pH 2.5–6.5 IEF in agarose gel and blotted onto nitrocellulose membranes (**b**) and *D. pteronyssinus* extract submitted to pH 4–6.5 IEF in agarose gel (**c**). Blot strips were tested with a pool of sera (A), a pool of sera inhibited by *H. aspersa* extract (B), a pool of sera inhibited by Der p extract (C), a pool of sera inhibited by Ota I extract (E), and a pool of sera inhibited by The p extract (F). D = Controls without serum. IgE binding to blotted proteins was detected by ^{125}I anti-human IgE in autoradiography.

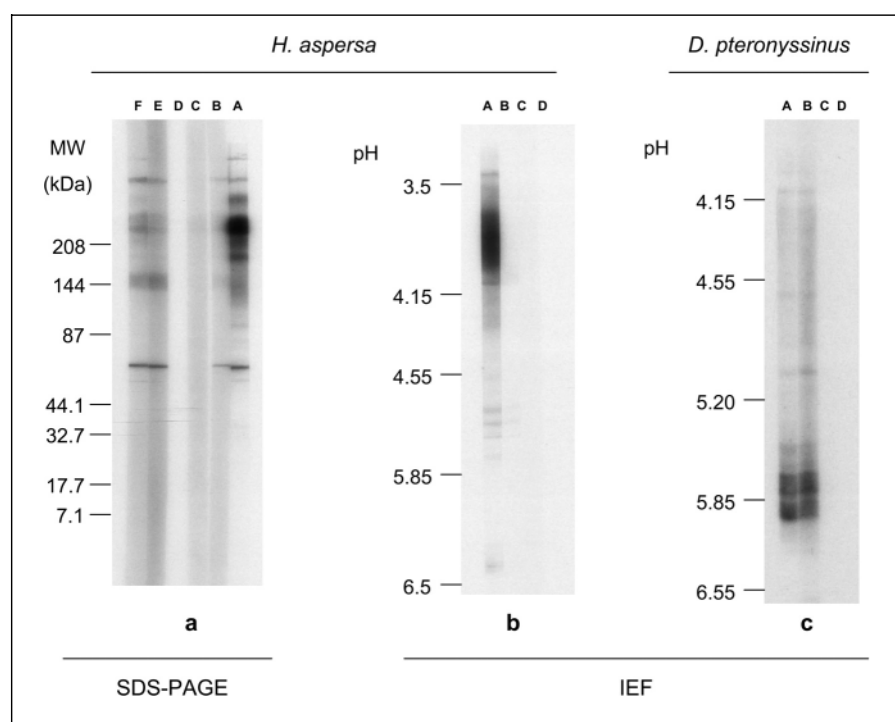
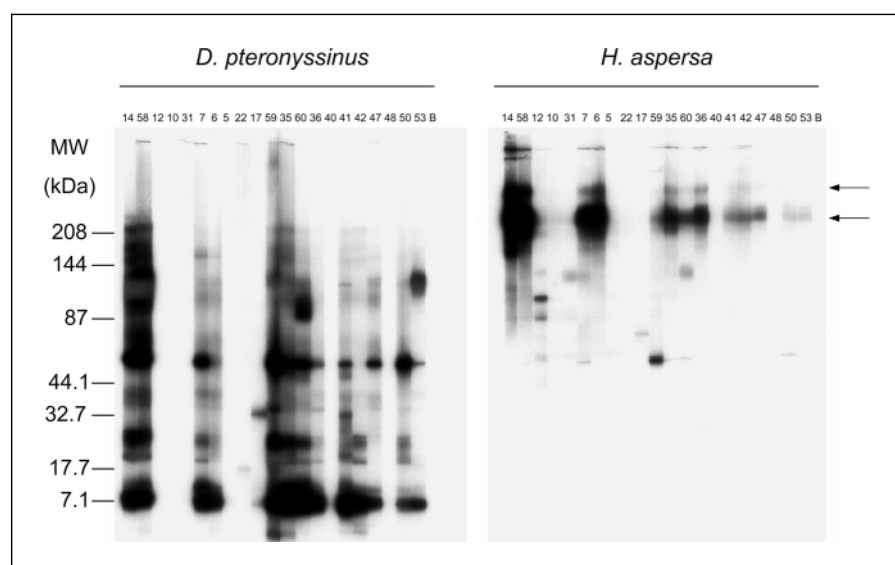


Fig. 3. IgE immunoblots with *D. pteronyssinus* and *H. aspersa* extracts submitted to 8–18% PAGE gradient and blotted onto activated PVDF membranes. Twenty-one patients/lane were tested for IgE binding. B = Control without serum. IgE binding to PVDF-blotted proteins was detected by ^{125}I anti-human IgE in autoradiography. Major recognized allergens are indicated (arrows).



Potency of the Extracts and Cross-Reactive Assays RAST Inhibition

H. aspersa extract inhibited the homologous RAST up to 96.9% but only 5.6% of the *D. pteronyssinus* RAST (fig. 6a). *D. pteronyssinus* extract inhibited the homologous RAST up to 89.9% (not shown), the Hel a RAST 72.6% and the *P. borealis* RAST up to 91.9% (fig. 6a). In each of the 6 snail-reactive patients, the results of

H. aspersa RAST inhibitions with *D. pteronyssinus* inhibitor extract differed significantly from those obtained in the serum pool (fig. 6b).

Immunoblot Inhibition

H. aspersa (fig. 2a, b, lanes B) and *D. pteronyssinus* extracts completely inhibited the homologous blots (fig. 2c, lane C), and the latter also the one from *H. aspersa*

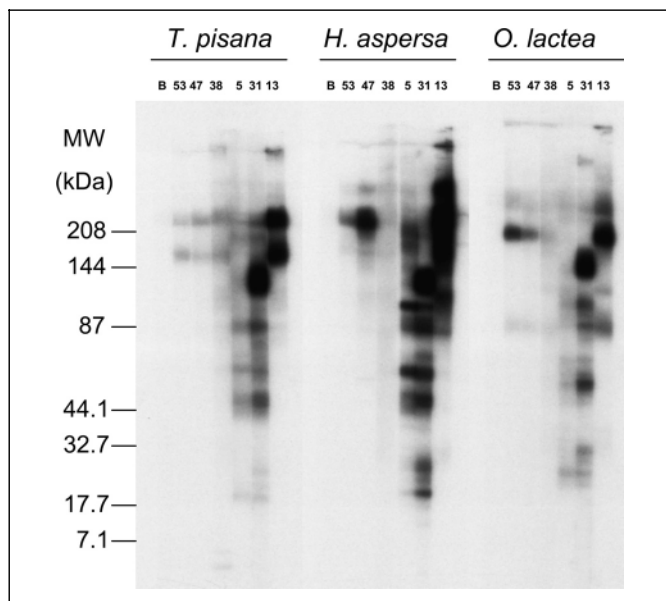


Fig. 4. IgE immunoblots with *T. pisana*, *H. aspersa* and *O. lactea* extracts submitted to 8–18% PAGE gradient and blotted onto activated PVDF membranes. Six snail-reactive patients (53, 47, 38, 5, 31 and 13) were tested for IgE binding. B = Control without serum. IgE binding to PVDF-blotted proteins was detected by ^{125}I anti-human IgE in autoradiography.

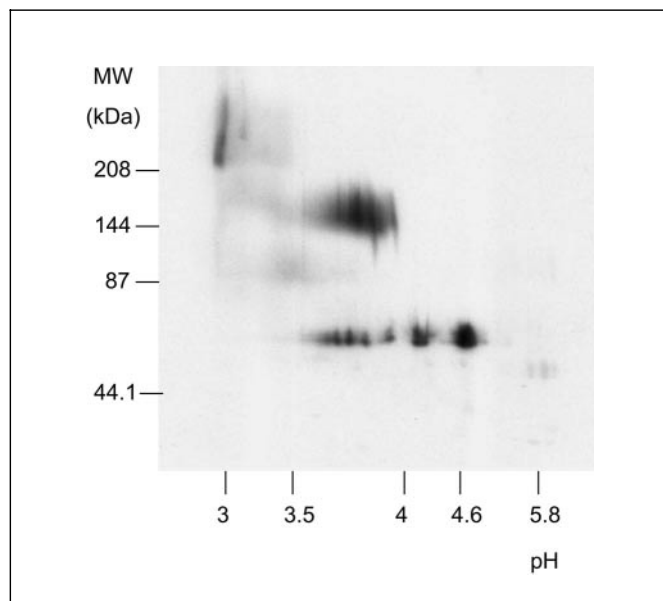


Fig. 5. Two-dimensional IgE immunoblot with *H. aspersa* extract firstly submitted to pH 2.5–6.5 IEF followed by 8–18 % PAGE gradient. A pool of 15 sera was tested for IgE binding. IgE binding to protein spots was detected by ^{125}I anti-human IgE in autoradiography.

Table 3. Allergens to *D. pteronyssinus*, *T. pisana*, *O. lactea* and *H. aspersa* recognized by SDS-PAGE

Der p	The p	Ota l	Hel a	
MW	MW	MW	MW	recognition
kDa	kDa	kDa	kDa	(n = 21 patients)
	>208	>208	>208	13
	>208	>208	>208	18
208	208	208	208	2
	194.5		194	2
171	146–186	132–171	171	5
151.5			134–150	5
			116.5	1
108.5			108.5	5
		85	85	9
	74		71–76.5	2
	61.5		62	4
58			59.5	6
37.5			37	1
29			28.5	
27		27		
24.5	25		24.5	
21.5	21.5		21	

(fig. 2a, b, lanes C). The contrary was not found, i.e. *H. aspersa* did not inhibit the *D. pteronyssinus* blot (fig. 2c, lane B). *O. lactea* and *T. pisana* extracts extensively inhibited the intensity of serum IgE recognition in *H. aspersa* immunoblots (fig. 2a, lanes E and F).

A detailed discussion of the results related with the 6 snail-reactive patients is important, as they present different immunological patterns regarding the snail/mite cross-reactivity.

Patient 5

Cross-sensitization between snail and mite was revealed, as the latter inhibits 16.4% of the former RAST, without primary sensitization to mite (specific IgE to Der p <0.35 kU/l). Patient 5 also presented complex spectrotypes for *H. aspersa*, suggesting a primary sensitization to snail.

Patient 53

This patient presented a simple spectrotpe for snail, recognizing one major allergen >208 kDa. Mite extract did not inhibit the snail RAST, showing that clinical response to snail is possible without detectable cross-sensitization to mite.

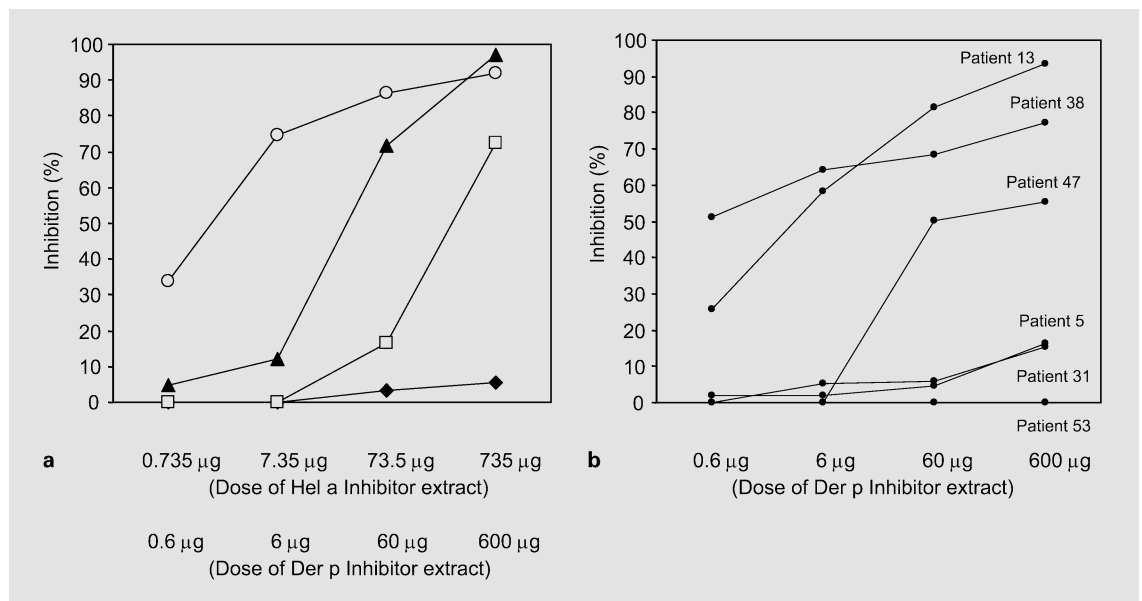


Fig. 6. RAST inhibitions. **a** Der p extract inhibiting Pan b RAST (○) and Hel a RAST (□). Hel a extract inhibiting Hel a RAST (▲) and Der p RAST (◆). **b** Der p extract inhibiting Hel a RAST from six snail-reactive patients.

Patients 13, 38 and 47

These patients represent the general hypothesis of primary sensitization to mite followed by extensive cross-reaction with snail. This evidence is shown by a significant inhibition of the *H. aspersa* RAST (93.4, 55.4 and 77.3%, respectively) to *D. pteronyssinus* extract. Patients 38 and 47 also presented simple immunoblot spectrotypes for the three snail species.

Patient 31

This patient showed evidence of cross-sensitization between snail and mite, with Der p extract inhibiting the Hel a RAST up to 15.2%. This patient presented complex immunoblot spectrotypes for *H. aspersa*, suggesting that snail may also represent, in this case, an important sensitizing agent.

Discussion

This study included 60 allergic outpatients with specific IgE to *H. aspersa* of class 2 or higher. Clinical examination discovered that 6 patients were having asthma after the consumption of snails, starting 15 min to 3 h after the ingestion. Specific IgE concentrations did not increase with the number of allergens recognized or allergic responsiveness. The relationship between mite and snail sensitiv-

ity is supported by the high prevalence of specific IgE and positive SPT for mites in these 60 patients.

RAST inhibition assays suggest that there is a high homology between commercial Cap RAST and home-made extract of *H. aspersa*, which is reflected by the high results of inhibition (96.9%). *D. pteronyssinus* extract also presented a high homology with Cap RAST allergens (89.9% of inhibition). Nevertheless, 3 patients (22, 40 and 48) did not show IgE to *H. aspersa* in blots, which is probably due to incomplete efficiency of the extraction and to the rather low sensitivity of the blotting technique. Nevertheless, IgE recognition in blots appeared to be quite specific when related to RAST determination, as 16 sera without specific IgE for *H. aspersa* (RAST <0.35 kU/l) did not present IgE recognition in snail blots.

In IEF and SDS-PAGE, *D. pteronyssinus* extract proved to cross-react with *H. aspersa* strongly: Hel a blots were totally inhibited and RAST in up to 72.6%. In contrast, snail extract proved much less cross-reaction towards mite, with no detectable inhibition of *D. pteronyssinus* spectrotypes in immunoblots and a low inhibition of the mite RAST (5.6%), suggesting that mite is the main sensitizing agent in the cross-reaction. This hypothesis is also supported by the fact that in this research very few patients were found with positive SPT or specific IgE to snail among the outpatients not sensitized to mites. A close example of cross-reaction was reported by Fer-

nandes et al. [37], referring IgE reactivity to the major shrimp allergen, tropomyosin, in house-dust-mite- and/or cockroach-allergic subjects not previously exposed to shrimps, apparently through a primary sensitization to mite and/or cockroach tropomyosins.

Five patients (9, 11, 15, 17 and 24) with specific IgE to *H. aspersa* did not present recognition in Hel a blots but showed a positive SPT for snail, which may be due to incomplete efficiency of extraction or transfer to blotting membranes.

Previously, cross-reactions between inhaled and ingested allergens have also been described in pollen and food allergy, which is mainly due to profilin, a common allergen preserved through different taxonomic lines during natural evolution, which is responsible for the cross-reactivity between plant-derived food and pollens [38]. Similar to the sensitization by IgE-inducing aeroallergens in food allergy, without clinical symptoms of pollinosis [39], allergy to snails seems to be mostly immunochemical in the study population, i.e. clinical reactions among mite-allergic patients may be absent. Also, with invertebrates such as crustaceans (shrimps, crabs, lobsters and crawfish), insects (cockroaches) and mollusks (squid, mussel, clam and snail), adverse food reactions have been found in allergic individuals due to the muscle protein tropomyosin. Sequence identities and similarities between *H. aspersa* and *D. pteronyssinus* tropomyosin are 65 and 79, respectively [40], but a 36-kDa protein from the *H. aspersa* extract, and the natural and recombinant purified tropomyosins were recognized by IgE antibodies in only 4 of 22 sera from patients reporting snail reactivity and showing specific IgE to *H. aspersa* above class 2 [41]. In our study, a 37-kDa protein from *H. aspersa* extract was also recognized by the IgE in only 1 out of 21 patients, and the two major allergens found are probably the heavy chains of myosin (~225 kDa), which were found in 13 and 18 of 21 patients, respectively.

Out of 56 patients with positive serum IgE determination to *D. pteronyssinus*, only 5 patients (13, 31, 38, 47 and 53) presented clinical reaction to snails. All of these 5 snail-reactive patients and patient No. 5 (without serum IgE to Der p) recognized at least one major allergen from *H. aspersa* extract >208 kDa, suggesting that the protein domains implicated in the allergic response may be included in the three-dimensional structure of snail myosin. The 6 snail-reactive patients showed a very close pattern of IgE recognition for the three snail species (*H. aspersa*, *O. lactea* and *T. pisana*), suggesting a narrow cross-reactivity between snails, which was verified in the *H. aspersa* immunoblot inhibition assay to *O. lactea* and

T. pisana extracts. Bands of 208 and >208 kDa were also recognized by 3 other subjects with specific IgE to *P. borealis* and to *D. pteronyssinus*, but not to *H. aspersa*. This indicates that the heavy chains of myosin may also be implicated in the cross-reaction between mollusks, crustaceans and arachnids.

Despite the existence of 50–60% sequence identity among vertebrate and invertebrate tropomyosins, a lack of IgE reactivity to vertebrate tropomyosins was found between meat-allergic subjects, associated with a low frequency of allergic symptoms to meats in crustacean-allergic subjects. This indicates that, in contrast to invertebrate tropomyosin, vertebrate tropomyosin is not important as a meat allergen [40].

The snail-reactive patients did not recognize the heavy chains of meat myosins (chicken, pork, rabbit, beef and horse), excluding the role of this high-molecular-weight protein in a possible cross-reaction between snails and meat.

Despite RAST inhibition studies showing significant inhibition of the IgE antibody reactivity to oyster, shrimp, lobster, crab and crawfish, the observed cross-reactivity of oysters and crustaceans does not always correlate with clinical response, suggesting that specific IgE to mollusks may not be enough to determine a clinical allergic response [40] similar to that with snails. In fact, sensitization to snails was frequently insufficient to predict a clinical reaction after ingestion.

Different factors possibly related with cross-reactivity may influence the progression to an asymptomatic sensitization or to a clinical allergic response. The repeated contact with the cross-reactive allergens, i.e. substances that have components with affinity to a single IgE antibody, is a prerequisite. This elicits immunological priming, with the allergen being recognized by virgin T and B cells. Then, the allergen will contact memory T and B cells. Finally, allergens contact the sensitized mast cells triggering the clinical response [42]. This three-step mechanism mimics what probably happens with the mite-/snail-sensitized subjects studied, because mites have a worldwide distribution and these outpatients are frequent snail consumers.

Most allergic patients present multiple sensitizations due to an increased immunologic responsiveness associated with atopy, and this process is linked at the T-cell level, since it is dependent on a Th2 response. The Th2 response to one allergen seems to facilitate further Th2 responses by promoting an environment that favors Th2 responses [42]. The study population seems to follow this pattern closely, by recognizing a large number of allergens from snail and mite separations, with elevated IgE levels and

marked SPT reaction. Further research on T-cell responses are in progress, which will possibly extend our understanding of specific patterns of response related with the clinical allergic reaction to snails.

The identification of molecular epitopes common in snail and mite remains to be evaluated in future studies. It will help to elucidate the factors involved in sensitization and clinical reactions. The high number and genetic

diversity of snail consumers in Southern Europe will certainly provide a fairly good sample.

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