

Description of a Novel Panallergen of Cross-Reactivity between Moulds and Foods

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The present investigation is undertaken to demonstrate a novel cross-reactivity between aeroallergens (moulds *fungi imperfecti*) and allergens from foods (spinach and mushroom *Agaricus bisporus*). We have performed a dual study in vivo and in vitro, in a population of atopic patients. Data from in vivo tests performed with spinach and mushroom have been statistically analysed. To the in vitro assays, mushroom and spinach extracts have been obtained, and sera from moulds allergic patients analysed by means of IgE-immunoblot assays. Inhibition experiments have been also performed to study a possible relation between proteins. Statistical analysis of data showed a relation between allergenicity to moulds (*Alternaria alternata*, *Cladosporium herbarum* and/or *Aspergillus fumigatus*), and positive skin prick tests with mushroom and/or spinach. The immunoblots performed showed that seven moulds allergic patients had a strong recognition of a protein with a molecular weight of about 30 kD present both in spinach and mushroom extracts, and by means of inhibition assays we could determine that these two proteins were related. This study demonstrates the existence of a new allergen responsible for cross reactivity between moulds and two frequently consumed foods, mushroom and spinach. We conclude that a novel cross-reactive allergen between aeroallergens and foods has been identified.

Keywords Moulds allergy, Mushroom, Spinach, Food allergy, Cross-reactivity, Panallergen.

INTRODUCTION

In the allergy field, panallergens are one of the most important lines of investigation, because these allergens can develop severe food allergic reactions in patients previously sensitized. These panallergens are also responsible for the progressive increase of prevalence of food allergy in the adult population (Breiteneder and Ebner, 2000; Breiteneder and Radauer, 2004).

The principal airborne allergens responsible for respiratory sensitization are pollens and mites, from which panallergens have been identified in both allergenic sources (Heiss et al., 1996; Reese et al., 1999; Ebner et al., 2001; Lagares et al., 2002). The association present between allergy to plant – derived food and pollen sensitization is one of the most common cross-reacting syndromes (Cuesta-Herranz et al., 1999; Vieths et al., 1998), mainly implicating birch (Hannuksela and Lahti, 1977), ragweed (Anderson et al., 1970), (Enberg et al., 1987), mugwort and grass (Hernandez et al., 1985) pollens. With RAST inhibition assays and immunoblotting, several common allergens responsible for the cross allergenicity between vegetable food and pollen have been described, which may lead to severe anaphylactic events and even death (Sampson et al., 1992; Savonius and Kanerva, 1993).

Moulds are another important airborne source of allergenic proteins (Achtze et al., 1995; Sarchez and Bush, 2001), which are responsible for respiratory allergic pathology, both allergic rhinitis and asthma (Michils et al., 1991; Kanerva et al., 1998; Helbling et al., 1999). The number of fungal species occurring in the environment is estimated to be at least 100,000, most of these can be propagated by airborne spores and therefore constitute potential respiratory allergens (Burge, 2002; Hoff et al., 2003). Over 80 genera of fungi have been associated with symptoms of respiratory tract allergy. Ascomycetes, Basidiomycetes, and Zygomycetes are the major fungal groups known to induce allergic reactions. Ascomycetes (belonging to the *fungi imperfecti* classification) include the greatest number of any fungal group, but only a few species, such as *Aspergillus fumigatus*, *Alternaria alternata*, and *Cladosporium herbarum*, have been investigated in depth. They belong to the most important known allergenic moulds, because they occur ubiquitously on all continents (Achatz et al., 1995; Sanchez and Bush, 2001; Hoff et al., 2003).

The worldwide prevalence of fungal allergy among atopic patients is estimated from 3% to 40%, depending on the allergic population studied, and the diagnostic methods and extracts used (Sanchez and Bush 2001).

With regards to cross-reactivity of allergens present in fungal sources, it is known that fungal allergic patients show sensitivity to multiple fungi (Verma et al., 2003; Weichel et al., 2003; Bisht et al., 2004), but few studies about cross-reactivity between these airborne allergens and sources without taxonomical relationship have been performed (Hoff et al., 2003). Recently, a fungal enolase has been identified as a highly conserved allergen among different fungal species, and also a protein of cross-reactivity between *Alternaria alternata*, *Cladosporium herbarum* and latex (Wagner et al., 2000; Chang et al., 2002).

Continuing in this direction, our group described the implication of fungal proteins in food allergy pathology, with a description of a new cross-reactive allergen present in *Alternaria alternata* and *Cladosporium herbarum* and two frequently consumed foods, spinach and mushroom *Agaricus bisporus* (Herrera et al., 2002). Later, the results of Dauby et al. (2002) supported our data.

Currently, our investigation seeks to provide a better understanding of this new cross-reactive protein between *fungi imperfecti* moulds and proteins present in foods. Our findings could help allergologists to make a more exact diagnosis and, more importantly, to anticipate and to prevent possible severe allergic reactions.

MATERIAL AND METHODS

Study Design and Patients Selection

Studying the possible relationship between moulds allergy and sensitization to mushroom and spinach, we have performed a dual study in vivo and in vitro, in an atopic patient population. From this group, 46 atopic patients were selected at random from outpatients referred to the Allergy Departments of Hospital Gregorio Marañón and Clínica Puerta de Hierro in Madrid. They were separated in two groups depending on the airborne sensitizations. Patients sensitized to moulds (*Alternaria alternata*, *Cladosporium herbarum*, and / or *Aspergillus fumigatus*) constituted the problem group (n = 33), and patients without moulds allergy but mites and / or pollens sensitization were the control population (n = 13).

The majority of the patients included in the study were between 10 and 30 years old; only one patient was older than 30 (44 years), and three patients were younger than 10 (5, 7 and 8 years). The mean age of the total 46 patients, was 19.6 years old. Of the total 46, 18 patients were female (40.5%) and 28 patients were male (59.5%). In the problem group, eleven patients in treatment with specific immunotherapy against moulds were included. The mean age of these patients was of 13.27 years old; all of them were asthmatic patients sensitized to *Alternaria alternata*. We gathered several data to posterior analysis: airborne sensitizations, clinical manifestations due to moulds, presence or not of specific immunotherapy against moulds, and several serologic data about total and specific IgE.

In Vivo Studies

Skin prick tests (SPTs) were performed with commercially available extracts (CBF LETI. Madrid. Spain) of common inhalant allergens (house dust mites, moulds, and trees, grasses and weeds pollens), and prick-by-prick tests with raw spinach and mushroom (*Agaricus bisporus*) were performed. Histamine hydrochloride and saline were used as positive and negative controls, respectively. A wheal diameter 3 mm greater than the negative control was considered a positive response. We performed specific provocation tests, nasal, ocular, or bronchial (depending on the symptoms of each patient), to determine the etiologic role of moulds in the allergic symptoms of the patients.

Sera of patients were stored at -20°C to further in vitro analysis.

IN VITRO STUDIES

Serologic Determinations

The level of total and specific IgE was measured with the Pharmacia CAP System (Pharmacia Diagnostics, Uppsala, Sweden) according to the manufacturer's instructions. Levels of specific IgE were measured to moulds *fungi imperfecti* (*Alternaria alternata*, *Cladosporium herbarum*, *Aspergillus fumigatus*), mushroom and spinach (when available).

Extracts

Taking into consideration several methods for food proteins extraction (Helbling et al., 1998, 2002), we performed the homogenization of each fresh material (raw spinach leaves and raw mushroom) at 50% p/v in buffered phosphate saline (PBS) containing 0,5 mM phenylmethylsulfonyl fluoride (PMSF). The homogenization was achieved by means of a domestic blender. The mixture was filtrated across glass wool and centrifuged at 4,500 g for 20 minutes to remove insoluble material. The supernatants were again centrifuged under the same conditions and put in storage at -70°C until further analysis.

SDS - PAGE and Specific IgE Immunoblot

SDS-PAGE of the different extracts was performed using a 16% acrylamide separating gel and 4% stacking gel. The gels were stained with Coomassie Brilliant Blue (Coomassie Brilliantblau R 250, Merck, Darmstadt, Germany) to detect proteins of the extracts, or electrophoretically transferred to nitrocellulose using a Multiphor II device (Pharmacia LKB) for 1 hour and 15 minutes. Nitrocellulose sheets were cut in strips and blocked with phosphate buffer saline (PBS) containing 5% of non-fat dry milk, overnight at 4°C. After blocking, the strips were incubated with patients or control sera diluted 1/5 in PBS containing 3% non-fat dry milk, and incubated overnight at 4°C. After washing in PBS -3% non-fat dry milk, the membranes were incubated with a monoclonal anti-IgE (Ingenasa, Madrid, Spain) diluted 1/1000 in PBS -3% non-fat dry milk, overnight at 4°C. After three washes in the same buffer, the membranes were incubated with a 1/2000 dilution of peroxidase-conjugated anti-mouse polyvalent immunoglobulins (Sigma Chemical CO., St Louis, MO, USA) for 2 hours at room temperature. Finally, the membranes were washed and bands visualized by Chemiluminescence method using ECL Western Blotting Detection Reagents (Amersham Biosciences. England).

IgE-inhibition Experiments

To investigate cross-reactivity between proteins present in spinach and mushroom in moulds-allergic patients, crossed inhibition assays were carried

out. We performed two inhibition assays with the serum of an asthmatic patient allergic to moulds (patient no. 22). In the first one, spinach was blotted on nitrocellulose, while in the second one mushroom extract was in the solid phase.

In both inhibition assays, aliquots of the serum (0.01 ml of a 1/20 dilution in incubation buffer) were mixed with 0.01 ml of the spinach and mushroom extracts and incubated overnight at 4°C. Thereafter, the aliquots were placed on the different splits of nitrocellulose membranes and the blot procedure was followed as described Previously.

STATISTICAL ANALYSIS

To analyse categorical data we performed a cross tabulation, and to inference determination we applied the Exact Fisher Test. For quantitative data we used the mean and standard deviation (sd), applying the t-test for inference. We did not assume equal variances and the level of significance was 5% ($\alpha = 0.005$). SPSS for Windows (v 11.5) was the statistical package used for analysis of data.

We searched possible significant relations between the fact to have a positive result in skin prick test (SPT) with mushroom or spinach or both, and several parameters: sex, age, sensitization to airborne allergens, allergic symptoms because of moulds allergy (subclinic pathology, mild symptoms of rhinitis or conjunctivitis, and asthma), specific immunotherapy against moulds, and total and specific IgE against moulds (*Alternaria alternata*, *Cladosporium herbarum*, *Aspergillus fumigatus*), mushroom and spinach.

RESULTS

Clinical Data and Skin Tests

Clinical data and results from skin prick tests with airborne allergens, spinach and mushroom are shown in Table 1. When we performed the statistical analysis of the different parameters in moulds allergic population, some interesting results were found. Thirty-one out of the 33 moulds allergic patients were sensitized to *Alternaria alternata*; 22 of these patients had a positive SPT with mushroom. Eight *Alternaria alternata* allergic patients failed to show a positive result ($P = 0.290$). Of the *Cladosporium herbarum* allergic patients, 12 (80%) had a positive SPT with mushroom; three patients allergic to this mould had a negative result ($P = 0.429$). Finally, with regards to *Aspergillus fumigatus* sensitization, the 77.7% of the patients allergic to this mould (seven patients) showed a positive result in skin tests with mushroom; two patients had a negative result ($P = 0.639$).

Table 1: Demographic and clinical data of patients (n = 46): A shows data from problem group (n = 33), and data from control population are shown in *table 1B* (n = 13).

1A: Problem group:									
Patient N°	Age (years)	Sex	Airborne sensitization			Symptoms	IT/years	P-P foods	
			Moulds	Others	Mushroom			Spinach	
1	12	F	AA, CH	G, O, Pl	A	0	+	+	
2	32	F	AA	G, O	SC	0	NT	NT	
3	26	F	AA	G, O, CA, Pl, Dpt, Df	SC	0	NT	NT	
4	28	M	AA	G, O	RC	0	+	+	
5	19	M	AA	Dpt, Df	RC	0	NT	NT	
6	10	M	AA	G, O, CA, Dpt, Df	A, RC	0	+	+	
7	14	F	AA	G	SC	0	+	-	
8	10	F	AA, CH, AF	G, Pl, CA, Ch, Dpt, Df	A	0	++	++	
9	13	M	AA	G, O, CA, Ch	A	4	-	-	
10	15	M	AA, CH, AF	G, O, CA, Ch	A	4	+	+	
11	11	M	AA		A	4	+	-	
12	15	F	AA, CH		R	5	+	+	
13	12	M	AA	G, O, Pl, CA, Ch	A	3	++	++	
14	14	M	AA, CH, AF	G, O, Dpt, Df	A	6	+	-	
15	10	F	AA, CH	G, O, Ch	A	0	+	-	
16	15	M	AA	G, O, Ch	A	5	+	NT	

17	5	M	CH	O, Dpt	SC	0	-	-	-
18	12	M	AA, CH, AF	G, O, CA	A	2	-	+	-
19	14	M	AA, CH, AF	G, O, CA, Ch	A	0	++	+	+
20	16	F	AA, CH, AF	G, O, Pl, Ch, CA, Dpt, Df	A	0	+	-	NT
21	26	F	AA	Dpt, Df	SC	1	++	+	++
22	10	F	AA, CH		A, RC	0	++	+	++
23	27	M	AA		R	0	+	+	+
24	30	M	AA, CH	G, O, Pl, CA, Dpt, Df	A, R	0	++	+	++
25	20	M	AA	G, O, Pl, CA, Dpt, Df	A, R	0	+	+	+
26	31	M	AA	G, O	A, R	0	+	+	+
27	23	F	AA	G, O, CA	SC	0	-	-	-
28	44	M	CH, AF	G, O, CA, Df	A	0	-	-	-
29	16	M	AA, CH, AF	Dpt, Df	A, R	4	++	+	+
30	7	M	AA	G, O, Ch	SC	0	-	+	-
31	8	F	AA, CH, AF	G, O, Dpt, Df	A	0	+	+	-
32	13	M	AA	G, O, CA, Ch	R	4	+	+	+
33	10	M	AA	G, O, CA	SC	0	-	+	-

(continued)

Table 1: Continued.

1BControl group:						
Patient N°	Age (years)	Sex	Airborne sensitization	Symptoms	IT/years	P-P foods
						Mushroom Spinach
34	41	M	O, CA, PJ	A, RC	4	- -
35	35	F	G, O	SC	0	NT NT
36	26	F	G, O, Dpt, Df	A	0	- +/-
37	31	F	G, O	RC	0	- -
38	23	M	G, O	A, R	1	- -
39	29	F	G, O, CA	SC	0	- -
40	46	M	G, O, CA	RC	0	- -
41	43	F	G, O	RC	0	- -
42	14	M	G, O, Pl, Ch, CA	RC	0	- -
43	12	F	G, Pl, Ch	A	0	- -
44	12	M	G, O, Pl, Ch	A	0	- +
45	13	M	G, O, CA	A	0	- +
46	11	M	G, Ch	RC	0	+ +

Sex: female (F), male (M).

Airborne sensitizations: Moulds: AA *Alternaria alternata*, CH *Cladosporium herbarum*, AF *Aspergillus fumigatus*.

Others: G grasses (included *Cynodon dactylon*), O *Olea europea*, Pl *Platanus acerofila*, CA *Cupressus arizonica*, Ch *Chenopodium album*, PJ *Parietaria judaica*, Dpt *Deimatophagoides pteronyssinus*, Df *Deimatophagoides farinade*.

Symptoms: A asthma, SC subclinic sensitisation, RC rhinitis and conjunctivitis, R rhinitis.

P – P: prick by prick.

NT: no performed test.

We did not find statistical significances when the rest of airborne sensitizations were analyzed, interestingly however, 8 out of the 9 mites allergic patients showed a positive SPT with mushroom ($P = 0.220$). We also found a relationship between mushroom sensitization and the symptoms of the patients. From the moulds allergic population, 20 patients had asthma symptoms, seven patients showed a subclinic sensitization (casual finding in skin prick test with battery of airborne allergens), and four patients had mild symptoms of rhinitis or conjunctivitis or both. Of the patients with asthma symptoms, 17 (85%) showed a positive skin prick test with mushroom; only three asthmatic patients failed to show a positive result ($P = 0.053$).

We also have analysed the variable specific immunotherapy. Eleven of the moulds allergic patients were in treatment with specific immunotherapy, nine of them showed a SPT positive with mushroom, and two had a negative result ($P = 0.666$).

With regards to skin prick tests performed with spinach in moulds allergic population, 13 out of the 22 patients with a skin prick test positive to mushroom had positive results also with spinach ($P = 0.693$). No statistical significances were observed between spinach sensitization and the different parameters analysed in the study, but we did determine that 12 out of 16 patients sensitized to *Cynodon dactylon* pollen showed a positive result in SPT with spinach ($P = 0.032$).

With regards to the control population, three patients had a positive SPT with mushroom; two of them had also positive results with spinach. Data of patients are shown in Table 1. None of the patients included in the study referred to symptoms after eating neither spinach nor mushroom.

Serologic Parameters

Data about total and specific IgE against moulds (*Alternaria alternata*, *Cladosporium herbarum* and *Aspergillus fumigatus*), mushroom and spinach in problem population are shown in Table 2. We found a relationship between an elevated level of total IgE and a positive result in skin prick test with mushroom. The mean value of total IgE in patients with positive mushroom skin prick tests was 633.70 KU/L (sd = 474.82), in contrast, patients with negative results in SPTs had a mean value of 252.46 KU/L (sd = 310.60). In the case of spinach, we did not find significant differences; the mean value of total IgE in patients with SPT positive was 543.06 (sd = 498.43), and 455.32 in patients with negative results (sd = 425.80).

We also analysed data about specific IgE against moulds. Eighteen patients sensitized to *Alternaria alternata* and with SPT positive with mushroom showed a mean value of specific IgE of 19.72 (sd = 25.24). In contrast, patients with a negative test had a mean value of 4.03 (sd = 5.41). No differences

Table 2: Results of total and specific IgE determinations (KU/L) in problem group (n=33).

Patient n°	Total IgE	Specific IgE				
		A. Alternata	Cl. Herbarum	Asp. Fumigatus	Mushroom	Spinach
1	NT	NT	NT	NT	NT	NT
2	NT	NT	NT	NT	NT	NT
3	1640,40	0,49	0,35	0,35	NT	NT
4	138	0,35	0,35	0,35	0,35	0,35
5	124	1,58	NT	NT	NT	NT
6	NT	NT	NT	NT	NT	NT
7	500	1,11	0,35	0,35	0,35	0,35
8	675	5,00	0,96	2,61	0,35	0,35
9	11,3	0,35	0,35	0,35	0,35	0,35
10	652	25,9	9,43	1,84	NT	NT
11	241	11,10	2,49	0,80	0,35	NT
12	940	49,70	1,22	1,97	0,35	0,35
13	562	14,10	1,26	2,76	0,35	6,31
14	1114	38,70	30,60	46,20	0,35	0,74
15	92,50	0,38	5,42	3,40	0,35	0,35
16	306	7,97	NT	NT	NT	NT
17	149	0,35	0,35	0,35	0,35	0,35
18	NT	4,36	0,56	0,55	0,35	0,37
19	652	25,90	9,43	1,84	NT	NT
20	622	26,60	4,78	5,25	0,36	0,35
21	9 (UI/mL)	0,35	0,35	0,35	0,35	0,35
22	878	10,50	0,35	0,35	NT	NT
23	54	1,33	0,45	0,67	NT	NT
24	NT	NT	NT	NT	NT	NT
25	1174,5	13,10	0,35	0,83	NT	NT
26	NT	NT	NT	NT	NT	NT
27	NT	NT	NT	NT	NT	NT
28	115	0,35	0,35	0,35	NT	NT
29	1218	103	15,20	10,06	NT	NT
30	422	2,49	0,35	0,38	NT	NT
31	275	8,17	0,92	4,62	0,35	NT
32	94,40	16,10	0,35	0,35	0,35	0,35
33	963	3,71	0,35	0,39	0,35	5,72

were found in data about specific IgE against *Cladosporium herbarum* and *Aspergillus fumigatus*. Finally, about specific IgE against mushroom and spinach we could only make determinations in 12 patients. We found four positive results to spinach, and only one to mushroom in a very low level (patient no. 20).

IN VITRO ASSAYS

Immunoblot Assays

In Figure 1 can be seen the immunoblot performed with mushroom (Figure 1A) and spinach (Figure 1B) extracts. This assay showed that seven moulds allergic patients strongly recognized a protein with a molecular

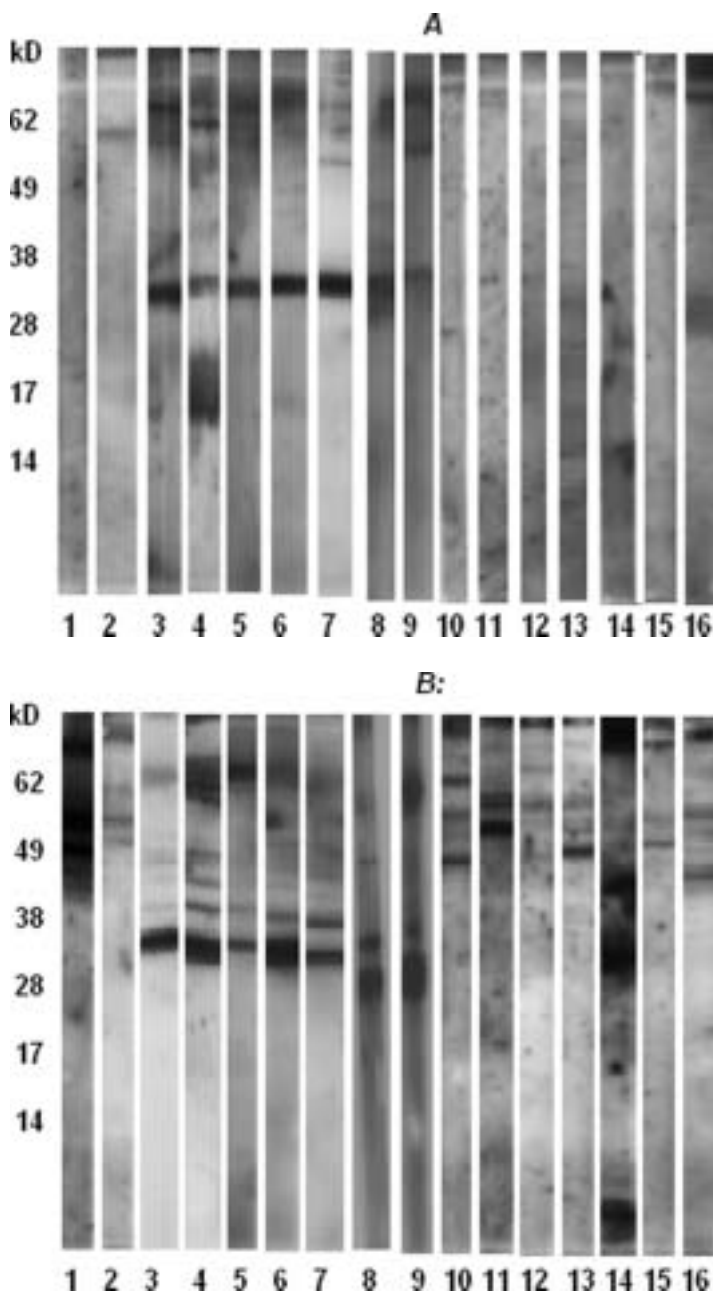


Figure 1: Immunoblot assays with mushroom (1A) and spinach (1B) extracts. Sera from two control patients (patients n° 34 and 35) are included in lanes 1 and 2. Results obtained in moulds allergic population are shown from lane 3 to 16: from lane 3 to 9 asthmatic patients (patients n° 1, 13, 15, 19, 20, 14 and 10), from lane 10 to 13 patients with mild symptoms (patients n° 2, 4, 5 and 7), and from lane 14 to 16 patients in treatment with specific immunotherapy (patients n° 11, 12 and 16). As we can see, a protein with a molecular weight of about 30 kD was strongly recognized by sera from seven asthmatic patients (from lane 3 to 9).

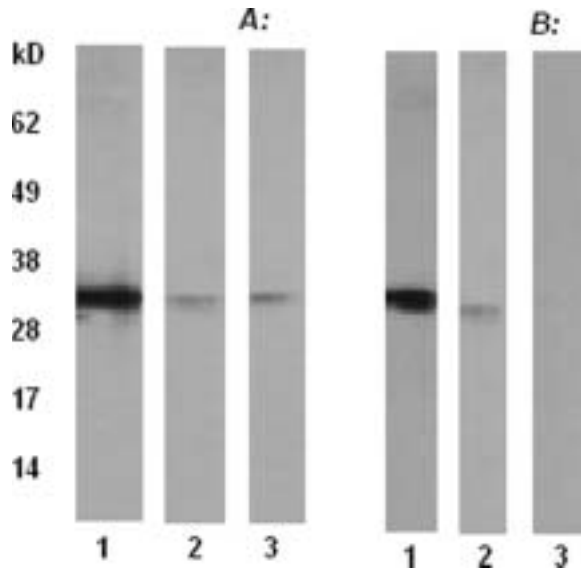


Figure 2: Immunoblot inhibition of IgE-reactivity against mushroom (2A) and spinach (2B) proteins. Blotts were performed with serum from patient n° 22 diluted 1/20 in incubation buffer, previously incubated with buffer (lane 1), mushroom extract (lane 2) and spinach extract (lane 3). Both mushroom and spinach extracts achieved a total inhibition of the 30 kD protein.

weight of about 30 kD in both mushroom and spinach extracts (patients no. 1, 13, 15, 19, 20, 14 and 10 in columns 3 to 9). All of them were asthmatic patients with allergy to moulds for several years. Patients with mild symptoms (patients no. 2, 4, 5 and 7 in columns 10 to 13) and patients treated with specific immunotherapy (patients no. 11, 12 and 16 in columns 14 to 16) did not show specific IgE against this protein. Two control patients (patients no. 34 and 35, columns 1 and 2) didn't show recognition of the 30 kDa protein. Clinical and serologic data of these patients are shown in Tables 1 and 2.

Inhibition Assays

As shown in Figure 2, both mushroom and spinach extracts were able to inhibit the binding of the patients' antibodies to the 30 kD protein present in mushroom and spinach extracts (column 3 in Figures 2A and 2B), indicating that some common epitopes must be present.

DISCUSSION

In the study of allergy pathology, panallergens are currently one of the most important lines of investigation. These cross-reactive allergens may lead to

severe food allergic reactions in sensitized patients (Sampson et al., 1992; Sanchez and Bush, 1993), and are one of the principal causes of the progressive increase of the prevalence of food allergy in the last decade, most importantly in the adult population. (Breiteneder and Ebner, 2000).

Cross-reactive allergens present in pollen and plant-derived food have been recognized as major causes of such symptoms (Heiss et al., 1996; Breiteneder and Ebner, 2000; Ebner et al., 2001; Lagares et al., 2002; Breiteneder and Radauer, 2004). It is well known that patients with birch pollen allergy frequently exhibit symptoms after eating certain fruits or vegetables (Lowenstein and Eriksson, 1983). Many plant-derived food contain proteins with high homology to the birch pollen allergens Bet v 1 (the major birch pollen allergen) and Bet v 2. Bet v 1 is a member of the pathogenesis-related proteins family belonging to PR-10 type protein, and Bet v 2 a profilin, one of the actin-binding proteins, and now recognized as a ubiquitous cross-reactive plant allergen. Allergens of other pollens have been also related with allergy to different fruits and vegetables, (i.e., *Chenopodium* and some grass pollens with beet, a vegetable of the *Chenopodiaceae* family) (Halmeupuro and Lowenstein, 1985; Wüthrich et al., 1990; Van Ree et al., 1992; Van Ree and Aalberse, 1993; Ebner et al., 1995).

Airborne moulds are another common source of respiratory allergic pathology with an estimated prevalence of 8% in adult population, and 20–25% in children with respiratory pathology (Sanchez and Bush, 2001; Burge, 2002). Little data about cross-reactive syndromes is available Bisht et al., 2004; Chang et al., 2002; Dauby et al., 2002; Herrera et al., 2002; Hoff et al., 2003; Verma et al., 2003; Wagner et al., 2000; Weichel et al., 2003.

Our data shows a relationship between allergenicity to moulds (*Alternaria alternata*, *Cladosporium herbarum*, and *Aspergillus fumigatus*), and in vivo sensitization to mushroom, demonstrated by SPT. When we analysed clinical data, we saw that the 85% of the patients with a positive skin prick test with mushroom were asthmatic patients ($P = 0.053$). Statistical analysis showed also a relationship with an elevated level of total IgE.

Immunoblot assays showed that 7 of the 22 mould allergic patients selected for in vitro analysis had a strong recognition of a protein with a molecular weight of about 30 kDa present both in spinach and mushroom extracts (as we can see in Figures 1A and 1B). All of these patients were mould allergic patients with clinical symptoms of asthma for several years. Patients with mild symptoms to mould allergy and patients in treatment with specific immunotherapy didn't show specific IgE against the 30 kD protein. Therefore, the data obtained from the in vitro study supported those previously obtained from the statistical analysis.

Only one asthmatic patient in treatment with specific immunotherapy showed recognition of the 30 kDa band in both extracts (patient no. 14, column 8 in Figures 1A and 1B). This patient was in treatment for six years with a progressive improvement of the clinical situation but also with a progressive

increase of specific IgE levels during the treatment. None of the control patients showed recognition of the 30 kDa protein, neither in spinach, nor in mushroom.

Therefore, our data clearly demonstrates the existence of an association between airborne moulds allergy (*Alternaria alternata*, *Cladosporium herbarum*, *Aspergillus fumigatus*), and sensitization against two foods without a taxonomical relationship, a vegetable of the *Chenopodiaceae* family, and mushroom *Agaricus bisporus*, a complex fungi belonging to *Basidiomycete* family. By means of inhibition assays we have demonstrated a relationship between the 30 kD protein present in spinach and mushroom extracts, showing that some common epitopes must be present in these different allergenic sources (see Figure 2). An interesting fact is that this protein present both in spinach and mushroom extracts, has a molecular weight similar to the major allergens of *Alternaria alternata* (*Alt a 1*) and *Cladosporium herbarum* (*Cla h 1*). More studies will be performed in order to study a possible relationship.

To conclude, both the important implication of *fungi imperfecti* moulds in respiratory allergic pathology, and the importance of the panallergens as causal agents of severe allergic food reactions, justify the study of cross-reactive allergens present in these airborne allergens. A more in depth knowledge of this 30 kD protein is necessary in order to improve the diagnosis, and, more importantly, to anticipate the possible development of severe adverse reactions due to cross-food allergy, as occur with other food allergic syndromes (Anderson et al., 1970; Hannuksela and Lahti, 1977; Heiss et al., 1996; Cuesta-Herranz et al., 1999; Reese et al., 1999; Breiteneder and Ebner, 2000; Ebner et al., 2001; Lagares et al., 2002; Breiteneder and Radauer, 2004; Vieths et al.,).

With our findings we can conclude that a relationship between airborne moulds (*Alternaria alternata*, *Cladosporium herbarum*, *Aspergillus fumigatus*), spinach and mushroom is present, which supports our previous data (Herrera et al., 2002). We can also conclude that the presence of asthma as a clinical manifestation of moulds allergy could be a "predisposed factor" to develop these associated sensitizations to spinach and mushroom, and that specific immunotherapy could be a "protective factor" in these patients. Currently we are performing several in vitro assays to enhance the knowledge of this new cross-reactivity protein.

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ABBREVIATIONS

SPT-	skin prick test
SDS-PAGE	dodecyl-sulfate-polyacrylamide gel electrophoresis
PBS	sodium phosphate-buffered saline
SD	standard deviation

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