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SUBLINGUAL REACTIVITY TO rBET V1 AND rPHL P1 IN PATIENTS WITH ORAL ALLERGY SYNDROME

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Oral Allergy Syndrome (OAS) in patients with pollen-induced rhinoconjunctivitis is caused by specific IgE recognizing cross-reacting epitopes of fruits and plants, which were clearly shown *in vitro*, but failed to be demonstrated *in vivo* by cross-challenges in the target organs. Considering the hypothesis of degradation of such epitopes in natural extracts, challenges with recombinant pollen allergens were done to evaluate the reactivity of the oral mucosa in OAS patients. Seventeen patients with OAS and rhinitis from birch (10) and grass pollen (7) and 10 non-atopic controls were studied by skin prick tests (SPT), allergen specific nasal challenges (ASNC) and allergen specific sublingual challenges (ASSC) with birch and timothy extracts and with rBet v1 and rPhl p1 at increasing concentrations from 1 to 1000 mcg/ml. None of the healthy subjects in the control group had any positive test for birch and timothy extracts or for recombinant allergens. In the OAS group the following results were observed: SPTs with recombinant allergens were positive in all patients, mostly at 10 mcg/ml concentration; ASNC with rBet v1 were positive in all patients, mostly at 100 mcg/ml; ASSC with natural pollen extracts were positive in only 2 of 17 patients, but in 15 of 17 with rBet v1 and rPhl p1, mostly at 500 mcg/ml and 1000 mcg/ml. ASSC with rBet v1 and rPhl p1 were positive with a mean concentration of 677 and 533 mcg/ml, respectively. The results of sublingual challenges with rBet v1 and rPhl p1 showed the *in vivo* cross-reactivity between pollens and foods in patients with OAS, but high concentrations of the recombinant allergens were needed to reproduce oral symptoms, thus explaining the failure of challenges performed with natural extracts, which have concentrations of major allergens lower than 50 mcg/ml. This indicates that sublingual mucosa is much less reactive to allergens than other surfaces, such as skin and nasal mucosa, probably because of its anatomic and immunologic peculiarity.

Oral allergy syndrome (OAS) is a condition characterized by oral swelling and itching after the contact of specific foods with the oral mucosa (1). In most cases this syndrome occurs in patients with pollen-induced rhinoconjunctivitis when eating fresh fruits or vegetables (2-4). The etiology of OAS is generally attributed to cross-reactivity between some food allergens and some inhalant allergens. The contact with the inhaled allergen leads to sensitization of upper and lower airways, with a production of specific IgE cross-reactive to food allergens, which, in turn, are responsible for the oral

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symptoms (5). Some authors have also described the occurrence of OAS preceding respiratory symptoms or even without them (6). RAST-inhibition experiments have confirmed that IgE can recognize cross-reacting epitopes of fruits and plants (7) and molecular biology techniques allowed the detection of allergens considered responsible for the cross-reactions (8-10).

In the attempt to confirm the cross-reactivity between foods and pollens in an *in vivo* study, in a previous work we performed a series of cross-challenges in patients with OAS, but both oral challenges with pollen extracts and nasal challenges with homogenized foods were negative (11). Taking into consideration the instability of cross-reacting allergens (12) this result could be attributed to the insufficient presence of such allergens, caused by degradation during the method of commercial extraction, or to cleavage of the endogenous proteases. *In vitro* high sensitivity and specificity of recombinant pollen allergens were described by several authors (13-16), and *in vivo* evaluation thus far includes skin prick tests with rBet v1 and rBet v2 (17), conjunctival provocation tests with rBet v1 isoform (18), and recently also specific nasal challenges with rBet v1 (19-20).

We evaluated the reactivity of the oral mucosa to pollen allergens in patients with allergic rhinitis and OAS using recombinant allergens, which are stable and standardized, in order to overcome the possibility of natural pollen degradation.

MATERIALS AND METHODS

Study design

We studied 17 patients with allergic rhinitis (9 males, 8 females, mean age 23.1 years), 10 with birch pollen rhinitis and a history of oral symptoms due to apple or celery and 7 with grass pollen rhinitis and a history of oral symptoms due to tomato or kiwi, and 10 healthy controls (6 males, 4 females, mean age 23.4 years). Diagnosis of allergic rhinitis was based on typical clinical history (lasting at least 2 years), skin prick test (SPT) positivity and RAST positivity to pollen allergens. The characteristics of the patients are summarized in Table I. Diagnosis of OAS was based on clinical history, prick + prick test positivity to the fresh food and oral open provocation test (20). During the study, patients were not allowed to use either antihistamines or topical and systemic steroids for at least one month before the investigation. The study was approved by the Ethical Committee of the University of Perugia, all patients and controls gave oral and written informed consent.

SPTs with pollen extracts and recombinant allergens

SPTs were performed, according to international guidelines (21), with commercial extracts from birch and timothy pollen (Stallergénes, Milan, Italy) standardized in IR (Index of Reactivity). rBet v1 and rPhl p1 (Biomay, Wien, Austria) were diluted in 0.9% sterile sodium chloride solutions in concentrations of 1, 10 and 100 mcg /mL. Tests were carried out on the forearm, using positive (histamine 1%) and negative (saline) controls for comparison and were read after 20 minutes. A minimal distance of 3 cm between each test field was applied. Wheal and flares were pen-marked and then transferred by cellotape onto a sheet of paper; the results were calculated as the mean of the major diameter of the wheal plus its orthogonal and expressed as class 0 to ++++. SPT was considered positive if the wheal was greater than 3 mm and with a diameter similar or larger than the histamine wheal. SPT with recombinant allergens were considered positive when the wheal achieved the diameter of the respective commercial allergen extract.

Prick + prick with fresh food

All patients with positive history of reactions to foods were also studied with the prick + prick technique. The test was performed using fresh fruits or vegetables and compared with histamine and negative control wheal, with the same method of SPT.

Allergen-specific serum IgE

From all patients and controls, specific IgE to birch, timothy, rBet v1 and rPhl p1 were determined by the Immuno-CAP System (UniCap IgE, Pharmacia, Uppsala, Sweden).

Allergen Specific Nasal Challenge (ASNC) with natural pollen extracts and recombinant allergens

Patients were required to be completely symptom-free at the time of the study. They took no medication (topical or systemic antihistamines, topical or systemic corticosteroids, topical cromolyn) in the month preceding the study. On the first day a specific nasal challenge with the causative pollen was done (22). The ASNC was performed by spraying into a nostril the allergen extract of timothy or birch (Stallergénes, Milan, Italy) at increasing concentrations of 1, 10 and 100 IR, starting with the lower one. If no symptom appeared after 10 minutes, the subsequent concentration was administered. The challenge was preceded by the administration of the vehicle alone as negative control. The severity of symptoms (itching, sneezing, rhinorrhea and

obstruction) was graded as follows: 0= absent, 1= mild, 2= moderate, 3= severe.

After at least one week, the same patients underwent a second series of nasal challenges. In this occasion an ASNC with the recombinant allergen (rBet v1 or rPhl p1) was performed using the same method. The concentrations used for recombinant allergens were 1, 10, and 100 mcg /ml.

Allergen specific sublingual challenge (ASSC) with fresh foods

At the same visit of the prick + prick testing, a sublingual challenge with fresh foods was performed in all patients with a clinical history suggestive of OAS. A small amount of the fresh food had to be kept under the tongue until symptoms appeared (usually less than 1 minute). The severity of symptoms (itching, oedema and feeling of foreign body) was graded from 0 (absent) to 3 (severe).

Allergen Specific Sublingual Challenge (ASSC) with natural pollen extracts and recombinant allergens

The double blind placebo-controlled ASSC was performed with 250 mcl of control solution, followed by the pollen extracts at increasing concentrations of 1, 10, and 100 IR. The allergen had to be kept under the tongue until symptoms appeared (usually less than 1 minute). After at least one week, together with the ASSC with recombinant allergens, the same patients underwent a second series of sublingual challenges with 250 mcl of recombinant extracts (rBet v1 or rPhl p1) at increasing concentrations of 10, 100, 500 and 1000 mcg /ml.

RESULTS

Prick + prick with fresh food

In patients allergic to birch pollen the prick + prick with fresh foods showed a positive result to apple in 8 cases and to celery in 2 cases. Prick + prick with tomato and kiwi were positive, respectively, in 6 and one patient who was also allergic to grass pollen (Table I).

Allergen-specific serum IgE

Serum specific IgE to foods, birch, timothy, rBet v1, and rPhl p1 were negative in all control patients (data not reported) and positive in all patients for the same allergen evaluated by prick tests (Table I).

SPTs

SPT with birch, timothy, rBet v1 and rPhl p1 were negative in all control patients (data not reported). rBet

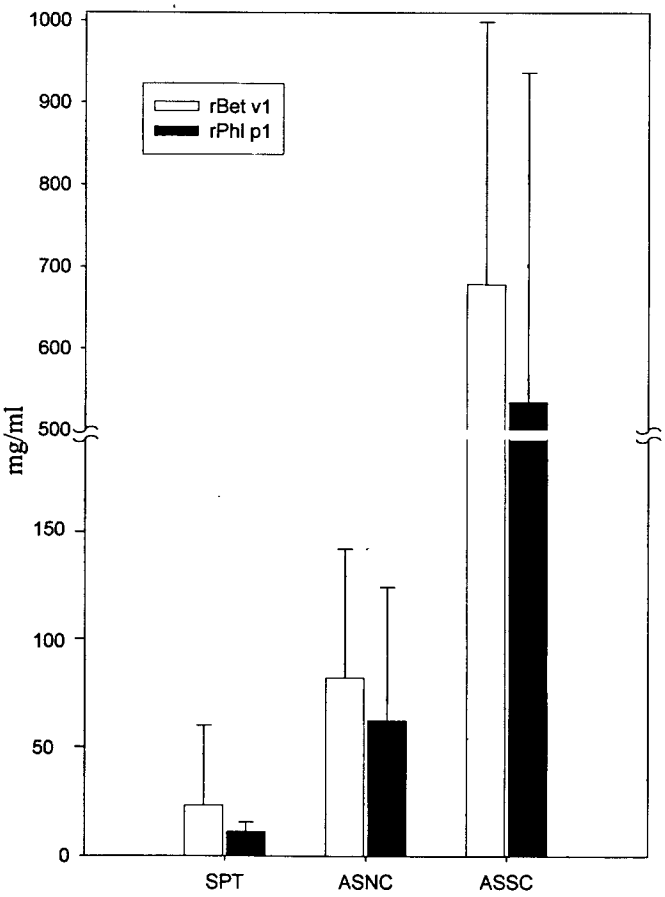

Fig. 1. *Comparison of the mean response to recombinant allergens of the oral mucosa with the mean response of skin and nasal mucosa. The mean concentrations of rBet v1 and rPhl p1 eliciting a positive response were respectively 18.1 ± 28.9 and 8.7 ± 3.40 mcg for SPT, 64 ± 46.5 and 48.5 ± 48.1 mcg for ASNC, and 677 ± 320.8 and 533 ± 403.3 mcg for ASSC, with significantly higher concentration for ASSC compared to ASNC ($p<0.01$) and to SPT ($p<0.005$).*

v1 showed a similar response to birch extract at 1 mcg/ml in one patient, at 10 mcg/ml in 8 patients and at 100 mcg/ml in one patient, corresponding to a mean concentration of 18.1 mcg/ml. rPhl p1 showed a similar response to timothy extract at 1 mcg/ml in one patient and at 10 mcg/ml in 6 patients, corresponding to a mean concentration of 8.7 mcg/ml. This is illustrated in Table II and Fig. 1.

Allergen Specific Nasal Challenge (ASNC) with natural extracts and recombinant allergens

All subjects in the control group showed no response to nasal challenges with natural extracts and

Table I. *Characteristics of the patients.*

Patient	Sex	Age	Prick + prick with fresh foods	Serum pollen specific IgE (KU/L)	Serum food specific IgE (KU/L)	Serum recombinant specific IgE (KU/L)	Oral challenge with foods
1	M	24	Apple ++++	Birch 79	Apple 13.4	rBet v1 >100	Apple +4
2	M	19	Apple ++++	Birch 21.3	Apple 4.5	rBet v1 17.9	Apple +3
3	F	20	Apple ++++	Birch 29.4	Apple 23	rBet v1 36.7	Apple +3
4	M	16	Apple +++-	Birch 11.5	Apple 15.1	rBet v1 18.6	Apple +4
5	F	22	Apple ++--	Birch 32.7	Apple 11.4	rBet v1 37.4	Apple +5
6	M	24	Apple +++-	Birch 39.5	Apple 8.7	rBet v1 6.17	Apple +3
7	M	29	Apple ++++	Birch 9.4	Apple 16.3	rBet v1 3.78	Apple +3
8	F	22	Apple +++-	Birch 7.5	Apple 3.5	rBet v1 5.7	Apple +4
9	F	30	Celery ++++	Birch 45.7	Celery 9.5	rBet v1 35.8	Celery +4
10	M	38	Celery ++++	Birch 29.4	Celery 12.3	rBet v1 19.3	Celery +4
11	F	27	Tomato ++++	Timothy 32.4	Tomato 22.9	rPhl p1 26.7	Tomato +4
12	F	20	Tomato +++-	Timothy 15.2	Tomato 3.7	rPhl p1 17.5	Tomato +3
13	M	21	Tomato ++--	Timothy 11.7	Tomato 4.15	rPhl p1 13.4	Tomato +3
14	F	18	Tomato +++-	Timothy 57.9	Tomato 6.4	rPhl p1 9.4	Tomato +4
15	M	23	Tomato +++-	Timothy 15.4	Tomato 16.4	rPhl p1 12.7	Tomato +3
16	M	19	Kiwi +++-	Timothy >100	Kiwi 7.5	rPhl p1 >100	Kiwi +4
17	F	21	Tomato ++--	Timothy 45.7	Tomato 17.3	rPhl p1 38.9	Tomato +3

recombinant allergens (data not reported). ASNC with rBet v1 were positive at 10 mcg/ml in 4 patients and at 100 mcg/ml in 6 patients, and ASNC with rPhl p1 were positive at 10 mcg/ml in 4 patients and at 100 mcg/ml in 3. Specific nasal challenges with recombinant allergens rBet v1 and rPhl p1 showed a similar response to natural extracts of birch and timothy with a mean concentration of 64 and 48.5 mcg/ml, respectively (Table III, Fig. 1).

Allergen Specific Sublingual Challenge (ASSC) with natural extracts and recombinant allergens

All subjects in the control group showed no response to sublingual challenges with natural extracts and recombinant allergens (data not reported). All patients had positive ASSC with fresh foods and negative ASSC with natural pollen extracts, except in 2 cases, one positive to birch and one positive to timothy (Table IV). ASSC with rBet v1 and rPhl p1 were positive in 15 of 17 patients: rBet v1 was positive at 100 mcg/ml in one patient, at 500 mcg/ml in 4 patients and at 1000 mcg/ml in 4 patients, while one patient was negative even to the maximum concentration of 1000 mcg/ml; rPhl p1 was positive at 100 mcg/ml in 2 patients, at 500 mcg/ml in 2 patients and at 1000 mcg/ml in 2 patients, one patient was negative to the maximum concentration of 1000 mcg/ml (Table IV). ASSC with rBet v1 and rPhl p1 were positive with a mean concentration of, respectively, 677 and 533 mcg/ml, significantly more elevated than SPTs ($p<0.005$) and ASNC ($p<0.01$) (Fig. 1).

DISCUSSION

Recognizing that symptoms arising at the contact between given fruits and vegetables and the oral and gastrointestinal mucosa are caused by sensitization to cross-reacting allergens naturally occurring in such foods and in pollens was a major advance in the understanding of the pathophysiology of allergy. In particular, one of the most common associations between pollinosis and food allergy – the so-called birch-apple syndrome – was clearly correlated to sensitization to the major allergen of birch, Bet v 1, which showed a 90% homology with the major allergen of apple Mal d 1 (23). Subsequently, a number of other cross-reactions involving pollens and

Table II. *Results of skin prick test.*

Patient	Skin prick test with commercial extract (IR)		Skin prick test with recombinant allergens (mcg/ml)			
		100		1	10	100
1	Birch	++++	Bet v1	+---	++--	++++
2	Birch	++--	Bet v1	----	++--	+++-
3	Birch	++++	Bet v1	+++-	++++	++++
4	Birch	++++	Bet v1	++--	++++	++++
5	Birch	++++	Bet v1	++--	++++	++++
6	Birch	++++	Bet v1	++++	++++	++++
7	Birch	+++-	Bet v1	+---	+++-	++++
8	Birch	+++-	Bet v1	+---	+++-	++++
9	Birch	++++	Bet v1	+--	++++	++++
10	Birch	++++	Bet v1	++--	++++	++++
11	Timothy	++++	Phl p1	++--	++++	++++
12	Timothy	+++-	Phl p1	+---	+++-	++++
13	Timothy	+++-	Phl p1	+---	+++-	++++
14	Timothy	+++-	Phl p1	+++-	++++	++++
15	Timothy	++--	Phl p1	----	++--	+++-
16	Timothy	++++	Phl p1	+++-	++++	++++
17	Timothy	++++	Phl p1	++--	++++	++++

Table III. *Results of nasal challenges*

Patient	Nasal challenge with commercial extract (IR)				Nasal challenge with recombinant allergens (mcg/ml)			
		1	10	100		1	10	100
1	Birch	Neg	Neg	+5	Bet v1	Neg	Neg	+4
2	Birch	Neg	Neg	+4	Bet v1	Neg	Neg	+5
3	Birch	Neg	+3		Bet v1	Neg	+4	
4	Birch	Neg	+4		Bet v1	Neg	+3	
5	Birch	Neg	Neg	+3	Bet v1	Neg	Neg	+3
6	Birch	Neg	+4		Bet v1	Neg	+4	
7	Birch	Neg	Neg	+3	Bet v1	Neg	Neg	+3
8	Birch	Neg	Neg	+4	Bet v1	Neg	Neg	+3
9	Birch	Neg	+4		Bet v1	Neg	Neg	+3
10	Birch	Neg	Neg	+3	Bet v1	Neg	+3	
11	Timothy	Neg	Neg	+4	Phl p1	Neg	Neg	+3
12	Timothy	Neg	+4		Phl p1	Neg	+3	
13	Timothy	Neg	Neg	+4	Phl p1	Neg	Neg	+3
14	Timothy	Neg	+4		Phl p1	Neg	+3	
15	Timothy	Neg	Neg	+3	Phl p1	Neg	+4	
16	Timothy	Neg	+3		Phl p1	Neg	Neg	+3
17	Timothy	Neg	+3		Phl p1	Neg	+3	

Table IV. *Results of oral challenge.*

Patient	Oral challenge with commercial extract (IR)				Oral challenge with recombinant allergens (mcg/ml)				
		1	10	100		10	100	500	1000
1	Birch	Neg	Neg	+2	Bet v1	Neg	Neg	+3	
2	Birch	Neg	Neg	Neg	Bet v1	Neg	Neg	Neg	+3
3	Birch	Neg	Neg	Neg	Bet v1	Neg	Neg	Neg	+4
4	Birch	Neg	Neg	Neg	Bet v1	Neg	Neg	+4	
5	Birch	Neg	Neg	Neg	Bet v1	Neg	+3		
6	Birch	Neg	Neg	Neg	Bet v1	Neg	Neg	+4	
7	Birch	Neg	Neg	Neg	Bet v1	Neg	Neg	Neg	Neg
8	Birch	Neg	Neg	Neg	Bet v1	Neg	Neg	Neg	+3
9	Birch	Neg	Neg	Neg	Bet v1	Neg	Neg	Neg	+3
10	Birch	Neg	Neg	Neg	Bet v1	Neg	Neg	+3	
11	Timothy	Neg	Neg	Neg	Phl p1	Neg	Neg	+3	
12	Timothy	Neg	Neg	Neg	Phl p1	Neg	Neg	+3	
13	Timothy	Neg	Neg	Neg	Phl p1	Neg	Neg	Neg	+4
14	Timothy	Neg	Neg	Neg	Phl p1	Neg	+3		
15	Timothy	Neg	Neg	Neg	Phl p1	Neg	Neg	Neg	+3
16	Timothy	Neg	Neg	Neg	Phl p1	Neg	Neg	Neg	Neg
17	Timothy	Neg	Neg	+3	Phl p1	Neg	+3		

foods was identified (24). One may reasonably suppose that the *in vitro* cross-reactivity may be reproduced *in vivo* using pollen extracts, but when we tested this hypothesis by performing oral challenges with birch and grass pollen extracts in patients with pollen-induced rhinoconjunctivitis and OAS from apple, celery, and tomato, the patients did not react to the challenge (11).

The availability of recombinant Bet v 1 to be used for SPT achieved a high diagnostic value in patients with the birch-apple syndrome and other related food allergies as well (25). Thus, in order to verify whether a low concentration of epitopes cross-reacting with foods may account for the lack of response to oral challenge with pollen extracts, we repeated the challenges in patients with pollinosis and OAS, and in healthy controls, using, along with commercial extracts, the recombinant allergens rBet v1 and rPhl p1, which served as material also for SPTs and nasal challenges.

In agreement with the results of a previous study (17), we found that SPT and nasal challenge with rBet v 1 and rPhl p 1 were negative in nonatopic controls and showed positive results in allergic patients, comparable to commercial extracts at concentrations of respectively 18.1 and 64 mcg/ml. Sublingual challenges with natural extracts were positive only in 2/17 patients, while sublingual challenges with recombinant allergens were positive in 15/17 patients with OAS, but with a mean threshold concentration of 677 mcg/ml for rBet v1 and 533 mcg/ml for rPhl p1, significantly more elevated compared with the mean concentrations needed to achieve positive SPT (18.1 and 8.7 mcg/ml) and nasal challenges (64 and 48.5 mcg/ml). This confirms the hypothesis that the use of recombinant allergens in place of natural pollen extracts directly in the target organ would be able to demonstrate an *in vivo* cross-reactivity between pollens and foods in patients with OAS. The higher amount of recombinant allergen needed to reproduce the typical symptoms of OAS, when compared with SPTs and nasal challenges, leads us to define that the oral mucosa has a higher threshold response than skin and nasal mucosa. Moreover, we should consider that the concentration of commercial extracts (100 IR) had an amount of natural major allergen lower than 50 mcg/ml (26, 27) and that the

negativity of oral challenges was probably due to the insufficient amount of natural allergen, lower than the oral threshold response. These low concentrations may also be secondary to the demonstrated degradation of allergens during the preparation of commercial extracts (28).

Another aspect to consider deals with SPTs in patients with OAS, who often show positive tests for several foods which are not commonly confirmed by the patients as really responsible for oral symptoms. In this situation oral challenges with fresh foods are usually carried out, but they are not standardized and sometimes difficult to perform. The data we obtained using rBet v1 and r Phl p1 show that sublingual challenges with these allergens, stable and standardized, may be used in the diagnosis of OAS related to birch and grass pollen allergy. Sublingual challenges with recombinant allergens, in fact, induced oral symptoms in 15/17 patients with OAS and no symptoms in 10 healthy controls, showing high sensitivity (88%) and specificity (100%).

Thanks to its safety, sublingual mucosa is an important site of administration for specific immunotherapy, which was used also in patients with pollinosis and OAS without important adverse reactions (29). The most common side effects reported during sublingual immunotherapy (SLIT) are oral and gastrointestinal symptoms (30). The high concentration of allergen needed to reproduce oral symptoms in the patients with OAS we observed, supports the safety of SLIT with recombinant allergens, nowadays used only in animal models (31).

In conclusion, this study clearly shows the *in vivo* cross-reactivity between pollens and foods in patients with OAS and indicates that sublingual mucosa is much less reactive to allergens than other surfaces, such as skin and nasal mucosa, probably due to anatomic and immunologic peculiarities. The low reactivity of sublingual mucosa to allergens adds new data to support the safety of oral mucosa as a site to obtain the immunological tolerance to allergens.

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