Immunoblotting in the diagnosis of cross-reactivity in children allergic to birch

Cudowska B[1]*, Kaczmarski M[1], Restani P[2]

[1] III Department of Paediatrics, Medical University of Białystok, Poland
[2] Department of Pharmacological Sciences, Faculty of Farmacy, State University of Milan, Italy

Abstract

Purpose: The scientific experiments with new immunological methods (immunoblotting, RAST inhibition) and isolation of recombinant allergens suggest structural similarities in the allergenic components responsible for cross-reactions. Immunochemical and molecular biology studies indicate that epitopes of major allergen (Bet v 1, Mal d 1) contain more IgE binding epitopes than minor allergens (Bet v 2, Mal d 2), what explained clinical importance of major birch and apple allergens, but it is individual. The important role in cross-reactivity play also proteins with low molecular weight; a potentially dangerous allergen is lipid transfer protein (LTP) inducing severe systemic reactions in allergic subjects. The recent studies indicate that the IgE cross-reactivity patterns and the clinical relevance is still not clear and that only some of patients with confirmed IgE cross allergy to Bet v 1 and Mal d 1 demonstrated clinical symptoms after ingesting of apple. The aim of study was to establish the pattern of cross-reactivity between major (Bet v 1) and minor (Bet v 2) birch pollen allergens and apple proteins in children allergic to birch using recombinant allergens and immunoblotting method.

Material and methods: The prospective study were carried out on the group of 13 children aged 4-16 years, referred to the IIIrd Department of Paediatrics in Białystok and outpatient clinic with clinical symptoms of food and inhalant allergy. Inclusion criteria to the study were: allergy to birch pollen recombinant allergens and apple, confirmed by presence of specific IgE in the sera of patients. The allergens from peel and pulp of apple and birch were separated and loaded onto the polyacrylamide electrophoretic gel and than transferred to membranes by western blotting. Antigen-IgE complex was detected using goat anti-human IgE antibodies labelled with alkaline phosphatase.

Results: Only few sera presented strong reactions in immunoblotting to birch pollen proteins with a molecular weight of 17-18 kDa, corresponding to the main birch allergen Bet v 1. Most of sera having positive reaction vs Bet v 1 showed cross-reactivity with Mal d 1. All sera recognized specifically the main allergen of apple peal Mal d 3 with molecular weight <10 kDa (Lipid Transfer Protein).

Conclusions: Immunoblotting method allows to verification of cross-reactivity recognized by presence of specific IgE. The nature of proteins responsible for sensitization can influence the spectrum of offending foods and the clinical features of allergic reactions.

Key words: cross-reactivity, birch-apple syndrome, immunoblotting, recombinant allergens, lipid transfer protein.

Abbreviations: IgE – immunoglobulin E; LTP – lipid transfer protein; RAST inhibition – radioallergosorbent test inhibition; SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Introduction

Patients allergic to birch pollen often also react with fruit and vegetables, such as apple and cross-reactivity of allergen-specific antibodies is a well-known phenomenon in food allergy [1,2]. Allergies to plant foods are based on cross-reactive IgE and adverse food reactions are mainly due to a specific family of related proteins. All these proteins seem to possess structural similarities. Proteins that share common epitopes with Bet v 1,

* CORRESPONDING AUTHOR:
III Department of Paediatrics
Medical University of Białystok
ul. Waszyngtona 17
15-274 Białystok, Poland
Tel: +48 85 7450704; Fax: +48 85 7423841
e-mail: becud@wp.pl (Beata Cudowska)

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the major birch pollen allergen, occur in other kinds of tree pollen, apples, stone fruit, celery, carrots and nuts [2]. The major cause of cross-reactivity between birch and apple is biochemical and immunological similarity between the major allergens, Bet v 1 and Mal d 1 [1]. Isolation and sequencing of the major allergen Mal d 1 from apple have shown a high degree of sequential homology with Bet v 1 and it has been demonstrated that Mal d 1 and Bet v 1 cross-react at T-cell level [3]. Bet v 1 belongs to class 10 of pathogenesis-related proteins. Other major allergen from apple (Mal d 2) has been identified as thaumatin-like proteins. In contrast to Bet v 1, two minor allergenic structures which sensitize about 10-20% of all pollen-allergic patients, are named profilins and cross-reactive carbohydrate determinants [2]. Cross-reactivity between birch pollen and apple has been demonstrated using allergen extracts in RAST inhibition, as well as immunoblotting [1]. A common observation of cross-reactivities has shown that a relatively high number of these seem to be without clinical significance [4]. More recently, a lipid transfer protein has been reported to be an important allergen in apple (Mal d 3). Lipid transfer protein (LTP), the major allergen in Rosaceae fruit in areas where the prevalence of birch pollen allergy is low, is a potentially dangerous allergen. Because of its extreme resistance to pepsin digestion, LTP probably reaches the intestinal tract in an almost unmodified form inducing severe systemic reactions in allergic subjects. In fact, lipid transfer proteins from apple showed a homology higher than 90%, which clearly explains immunochemical cross-reactivity in patients not allergic to birch pollen [5].

Food proteins and profilins of the Bet v 1 family are relatively sensitive to heat and can be easily cleaved by proteases. Serological assays and skin prick tests allow no distinction between symptomatic and asymptomatic patients. Positive serological assays should not be taken as indicators for a strict avoidance of foods which are tolerated [2].

Despite the increasing knowledge of cross-reactive structures and the role of recombinant allergens, it is still not clear the IgE cross-reactivity patterns and the clinical relevance [2,4]. According to the published data, sensitization to Bet v 1 or profilin is not always associated with IgE against specific foods. The correlation has been found between Bet v 1 and apple, peach, hazelnut in patients allergic to birch, but the actual question is, if patients allergic to apple demonstrate reactivity to Bet v 1 [2,6].

The aim of our study was to determine the pattern of cross-reactivity between birch pollen and apple in children allergic to birch using recombinant allergens and immunoblotting method. The correlation between immunochemical cross-reactivity to apple and birch was also established.

Material and methods

Patients

The prospective study were carried out on the group of 13 children aged 4-16 years, referred to the IIIrd Department of Paediatrics in Białystok and outpatient clinic with clinical symptoms of food and inhalant allergy. Inclusion criteria to the study were: allergy to birch pollen recombinant allergens (Bet v 1 and Bet v 2) and apple, confirmed by estimation of specific IgE in the sera of patients. Exclusion criteria were: chronic diseases of respiratory and digestive tract with different than allergic background, parasites infections, autoimmunological disorders.

Table 1. **Total and specific IgE in the investigated patients (N=13)**

No	Initials	Age (yrs)	IgE (IU/ml)	Specific IgE to birch allergens (class)			Specific IgE to apple (class)
				Birch	Bet v 1	Bet v 2	
1.	M.W.	14	65	3	3	0	2
2.	K.K.	4	>5000	3	3	3	2
3.	J.M.	12	3293	4	4	3	2
4.	M.R.	8	>5000	2	0	0	3
5.	J.Z.	2	2077	5	5	0	3
6.	P.L.	12	356	2	0	0	2
7.	J.B.	13	965	3	3	0	2
8.	M.W.	13	110	4	4	0	1
9.	J.S.	14	211	3	3	2	2
10.	L.M.	9	1081	3	3	0	4
11.	M.K.	4	141	6	6	0	4
12.	K.K.	13	792	4	4	0	3
13.	E.P.	16	>1000	3	2	0	2

Determination of total IgE and specific IgE antibodies

Serum samples (2 ml) were analyzed for concentration of total IgE and specific IgE antibodies to birch recombinant (Bet v 1 and Bet v 2) and apple allergens (Pharmacia Upjohn) with a fluoroimmunoenzymatic essay (UniCAP) according to the manufacture's instruction. The detection limit of the CAP system is 0.35 kU/L IgE; measurable specific IgE was defined as a positive test result if >0.7 kU/L. The results of total and specific IgE to birch and apple are presented in *Tab. 1*.

Immunoblotting

Immunoblotting has been performed in the cooperation with the Department of Pharmacology in the University of Milan in Italy with the procedures described bellow.

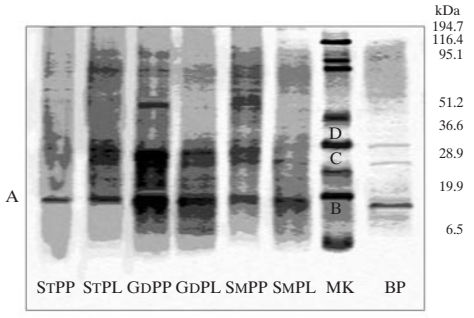
Apple

The apples were purchased from a greengrocer of Milan; they belonged to three varieties: Golden Delicious, Stark Delicious and Smith. Peel and pulp were separated, freeze-dried and suspended in Sample buffer:water (1:1, v:v) at the final concentration of 150 mg/mL. After a night at room temperature, samples were centrifuged at 10 000 rpm and 4°C for 20 minutes. The supernatant was collected and loaded onto the electrophoretic gel. Sample buffer contained 0.25M TRIS-HCl pH 6.8, 7.5% glycerol, 2% SDS, 5% β-mercaptoethanol.

Birch

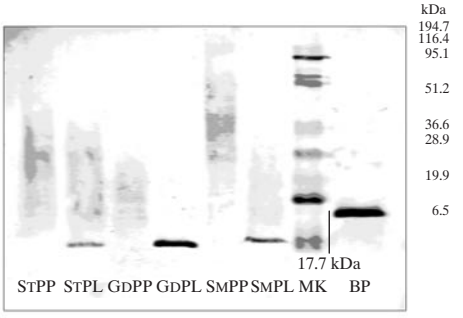
A birch solution for prick test (STALLERGENES SA) was diluted 1:1 (v:v) with Sample buffer and loaded onto a gel.

Figure 1. **SDS-PAGE of apple peel and pulp and birch pollen**



St – *Malus domestica* var. stark delicious; PP – pulp; Gd – *Malus domestica* var. Golden Delicious; PL – peel; Sm – *Malus domestica* var. Smith; MK – molecular weight marker solution; BP – birch pollen
Molecular weight:
A: 18.4 kDa; B: 17.7 kDa; C: 31 kDa; D: 35.4 kDa

Figure 2. **Reactivity vs birch pollen proteins 17-18 kDa in one of patient allergic to birch (membrane incubated with serum 11)**



St – *Malus domestica* var. stark delicious; PP – pulp; Gd – *Malus domestica* var. Golden Delicious; PL – peel; Sm – *Malus domestica* var. Smith; MK – molecular weight marker solution; BP – birch pollen

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Apple and birch proteins were separated on a gradient polyacrylamide gel with the following characteristics:

1 – Gradient running gel: 12-22% acrylamide; 0.11-0.20% bis-acrylamide; 0.36 M TRIS-HCl buffer pH 8.8; 35% glycerol; 0.1% SDS; 0.02% ammonium persulfate; and 0.15% N,N,N',N'-tetramethylenediamine (TEMED).

2 – Stacking gel: 3.5% acrylamide; 0.09% bis-acrylamide; 0.125 M TRIS-HCl buffer pH 6.8; 0.1% SDS; 0.02% ammonium persulfate; and 0.15% (TEMED).

3 – Running buffer: 25 mM TRIS, 0.19M glycine and 0.1% SDS (w/v), pH 8.8.

After the electrophoretic run (90 V at room temperature, for approximately 6 h) gels were dyed with Coomassie Brilliant Blue G-250. All materials and instruments were purchased from Bio-Rad (Richmond CA, USA).

Molecular Weight Marker Solution (prestained broad range, Bio-Rad) contained myosin (rabbit muscle; 194.7 kDa), β-galactosidase (Escherichia coli; 116.4 kDa), bovine serum albumin (95.1 kDa), ovalbumin (chicken egg; 51.2 kDa), carbonic anhydrase (bovine erythrocytes; 36.6 kDa), soybean trypsin inhibitor (soybean; 28.9 kDa), lysozyme (chicken egg white; 19.9 kDa) and aprotinine (bovine pancreas; 6.5 kDa).

Immunoblotting

After SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) membrane (PVDF; Immobilon P, Millipore, Bedford, MA, USA) by western blotting in a Trans-blot Electrophoretic Transfer Cell (Bio-Rad). The transfer buffer was 25 mM Tris, 193 mM glycine and 20% methanol. The membranes were blocked with 1% gelatin and washed three times with 0.25% gelatin solution (in 150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.05% Triton-X) to prevent non-specific adsorption of the immunological reagents. The membrane was then immersed in 10 mL of 0.25% gelatin solution containing 0.5 ml of serum from allergic children. Antigen-IgE complex was detected using goat anti-human IgE antibodies (Sigma Aldrich, Milan, Italy) labelled with alkaline phosphatase; the secondary antibody commercial stock was diluted 1/1000 (v:v) in 0.25% gelatin solution. After incubation for 4 h at room temperature with shaking, membranes were washed twice with 0.25% (2 min each) and once with Tris buffer solution (20 mM Tris and 0.5 M NaCl) for 5 min.

Finally, after incubation in bromochloroindolyl phosphate-nitroblue tetrazolium (BCIP-NBT) solution, an intense black-purple precipitate developed at the site of enzyme binding. The developing solution contained 15% BCIP and 30% NBT in alkaline phosphatase buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5).

Results

The electrophoretic pattern of apple pulp and peel belonging to the three selected varieties (*Malus domestica* var. golden delicious, stark delicious and smith) are shown in SDS-PAGE (*Fig. 1*). In parallel, the profile of pollen birch proteins is shown. The protein profile of apple samples is not well defined; in fact, several proteins are distributed in the range of molecular weights 6.5-95.1 kDa. Same proteins are well defined and among them the most abundant component (both in pulp and peel samples) presents a molecular weight of approximately 17-18 kDa.

The profile of birch pollen presents three major proteins, having molecular weights of approximately 18, 31, 35.5 kDa.

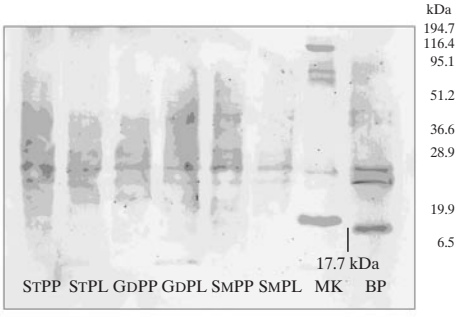
Circulating IgEs from the 13 sera included in this study were tested for reactivity against apple peel and pulp, and birch pollen allergens. The use of prestained Molecular Weight standard solution (broad range) allowed the identification of the proteins recognized by circulating IgEs (positive reaction in immunoblotting).

The main results can be summarized as follows:

1. Reactivity to birch pollen

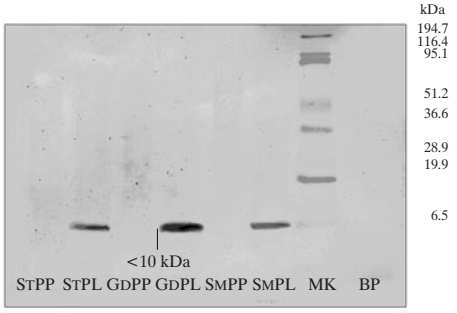
Only sera of 4 patients presented strong reaction to birch pollen proteins (*Fig. 2*). The protein recognized by these sera

***Figure 3*. Reactivity vs the allergens having molecular weight 17-18 kDa and two bands of approximately 30 kDa (membrane incubated with serum 5)**



St – *Malus domestica* var. stark delicious; PP – pulp; Gd – *Malus domestica* var. Golden Delicious; PL – peel; Sm – *Malus domestica* var. Smith; MK – molecular weight marker solution; BP – birch pollen

Figure 4. Reactivity vs major component of apple peel (Mal d 3 – Lipid Transfer Protein) (membrane incubated with serum 1)



St – *Malus domestica* var. stark delicious; PP – pulp; Gd – *Malus domestica* var. Golden Delicious; PL – peel; Sm – *Malus domestica* var. Smith; MK – molecular weight marker solution; BP – birch pollen

presented a molecular weight of approximately 17-18 kDa, corresponding to the main birch allergen Bet v 1. IgEs from the child 5 recognized in the birch pollen sample: a protein having molecular weight of 17-18 kDa (Bet v 1), and two bands of approximately 30 kDa (Bet v 6) (*Fig. 3*). Since allergy to birch pollen was the inclusion criterium for this study we must conclude that birch allergens contain conformational epitopes that loose their reactivity after denaturating processes (treatment with SDS and β-mercaptoethanol). Probably, only the sera from the most reactive subjects are capable to recognize denatured birch allergens.

2. Reactivity to apple peel proteins

All subjects presented reactivity against the major component of peel. Considering its molecular weight (<10 kDa), this protein can be identified as the known allergen Lipid Transfer Protein (Mal d 3) (*Fig. 4*). The reactivity against pulp proteins is characterized by a complex pattern of responses:

Serum 1: no reactivity

Serum 2: no significant reactivity

Serum 3: no significant reactivity

Serum 4: some reactivity to the peel allergen Mal d 1 (MW 17-18 kDa)

Serum 5: reactivity versus two allergens having molecular weights of app. 30 kDa

Serum 6: weak reactivity versus the allergen having molecular weight of 17-18 kDa (Mal d 1). The reaction is stronger versus apple Golden

Serum 7: reactivity versus the allergen having molecular weight of 17-18 kDa (Mal d 1). The reaction is stronger versus apple Golden

Serum 8: no reactivity

Serum 9: reactivity versus the allergen having molecular weight of 17-18 kDa (mainly in apple Golden) corresponding to the major birch pollen allergen (Bet v 1)

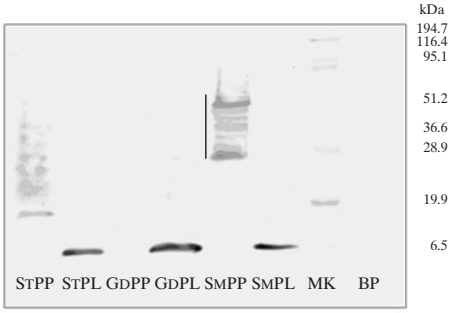
Serum 10: no significant reactivity

Serum 11: low reactivity versus the allergen Mal d 1

Serum 12: strong reaction versus Mad d 1 in the sample containing the apple var Smith (*Fig. 5*)

Serum 13: no significant reactivity.

***Figure 5*. Strong reactivity vs Mal d 1 in the sample containing the apple var. Smith (incubation with serum 12)**



St – *Malus domestica* var. stark delicious; PP – pulp; Gd – *Malus domestica* var. Golden Delicious; PL – peel; Sm – *Malus domestica* var. Smith; MK – molecular weight marker solution; BP – birch pollen

Discussion

Cross-reactivity between birch pollen and apple has been confirmed by analysis of recombinant proteins and using RAST inhibition and immunoblotting. The major cause of birch-apple syndrome is biochemical and immunological similarity between major allergens, Bet v 1 and Mal d 1 [1]. The epitopes of major allergens (Bet v 1, Mal d 1) show the highest IgE-affinity, but the different patterns of IgE-binding were also observed in individuals. Approximately 70% of patients who are allergic to birch pollen may experience symptoms after consumption of apple and fruit from family Rosaceae. Most of patients suffer from local symptoms at the site of the primary allergen contact (oral allergy syndrome) [2]. Some of them are also sensitized to minor birch pollen allergen; profilin Bet v 2, but recent studies suggested that profilin sensitization has little or not clinical relevance [2,7,8]. A possible reason for this fact is monosensitization to profilin or sensitization to other cross-reacting

structures in pollen and vegetables foods or to primary food allergens, such as LTP [2]. The recent studies indicated also that only 75% of patients with confirmed IgE cross-allergy Bet v 1 – Mal d 1, demonstrate clinical symptoms after ingesting of apple. This homology between allergens is clinically irrelevant because of cross-reactive carbohydrate determinants [9]. In our group of children, we observed positive oral food challenge to apple in seven of them; 4 sera showed reactivity to Mal d 1 and all to Mal d 3.

The next problem is that the presence of specific IgE to birch and apple in the sera of patients can not be considered as the predicting factor of clinical symptoms. As was previously described, sensitization to Bet v 1 is specific for birch and apple allergies, whereas sensitization to Bet v 2 is common in polysensitized patients. In the investigations carried out by Rossi et al., more than half of patients with a history of oral allergy syndrome after eating apple reacted to Bet v 1 [10]. In our study only few among 13 children allergic to birch presented in immunoblotting specific IgE capable to recognize birch pollen allergens; probably due to the denaturating processes used in this technique. The protein of birch recognized by the sera of 4 patients has a molecular weight corresponding to the main birch allergen Bet v 1 (17-18 kDa). As we have shown in the *Tab. 1*, all patients were allergic to birch according to the results of specific IgE. These results indicated that there is no correlation between these methods and that the presence of specific antibodies has not the clinical relevance. The great importance has also technical conditions of method. We supposed the influence of denaturating processes on the epitopes structure and the data obtained by other authors, concerning food allergens, confirmed these conclusions. The investigations carried out by Vieths and al. indicated that the proteins in the prick test solution appeared to be strongly degraded and that extraction procedures should be adapted to the specific source material [11].

Our results demonstrated that most of sera having positive reaction vs Bet v 1 showed cross-reactivity with Mal d 1, but some of patients showed reactivity to Mal d 1, even though the reactivity to birch was weak or absent. Detailed analysis of molecular surface areas performed by Holm et al. identifies potential epitopes for cross-reactive antibodies. A minimum of two epitopes would be necessary for cross-linking of receptor bound IgE in histamine release and skin test. The occurrence of limited epitope coincidence between Bet v 1 and Mal d 1 is in agreement with the observation that not all birch pollen allergic patients react with apple and that conformation of epitopes recognized by the IgE of the individual patients determines the degree of cross-reactivity [1].

Very interesting seem to be the results of reactivity to the apple protein with molecular weight <10 kDa, Mal d 3 (Lipid Transfer Protein). Our results showed that all sera, even these without reactivity in immunoblotting to birch, recognize specifically the main allergen of apple peel. LTP is the major allergen in Rosaceae fruit in areas where the prevalence of birch pollen allergy is low and is responsible for severe allergic reactions [5]. This protein show extreme heat stability and the results of investigations performed by Asero et al. suggest that LTP-hypersensitive patients with a history of severe reactions induced by apple should be advised to avoid unpeeled apple even after it undergone thermal processing [5]. Clinical observation of the investigated group of children indicated that only some of children demonstrated symptoms after ingestion of apple, but we didn't notice severe clinical reactions among them. The results of our investigations and the prevalence of sensitization to LTP is the indication for considering the spectrum of offending foods and dietetic restrictions, because of the risk of potentially life-threatening reactions relevant to heat stable and pepsin resistant proteins [12].

The recently studies indicated that the expression of Mal d 1 varies between different apple strains. Golden Delicious and Granny Smith apples have a high expression of Mal d 1 compared to Jamba and Gloster. It is with correlation of clinical symptoms occurred more often after ingestion of green apples than red ones. Hansen and al. described the seasonal variation in food allergy to apple. Although specific IgE against Golden delicious increased during season, neither skin test nor immunoblotting could confirm an increase in reactivity. The results of immunoblotting performed with the sera of our patients, showed stronger reactions vs apple Golden.

The analysis of the immunoblotting results in correlation to clinical observations of investigated children generally agreed that the diagnosis of food allergy must rely on the outcome of oral food challenge [13]. Because clinical aspect of cross reactivity was not the subject of this paper, we didn't compare the diagnostic accuracy of immunoblotting with clinical symptoms. But according to our experiences, we can suggest that immunoblotting is useful to verification of cross-reactivity recognized by the presence of specific IgE.

Acknowledgement

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