
Allergy to nonspecific lipid transfer proteins in Rosaceae: a comparative study of different *in vivo* diagnostic methods

Riccardo Asero, MD*; Gianni Mistrello, BSc†; Daniela Roncarolo, BSc†; Manuela Casarini, BSc†; and Paolo Falagiani, DVM†

Background: Lipid transfer proteins (LTPs) are the major allergens in patients sensitive to Rosaceae (apple, peach, apricot, cherry, plum, and pear) who are not allergic to birch pollen.

Objective: The purpose of this study was to find a sensitive, specific, and relatively easy method for detection of LTP-sensitive patients.

Methods: We studied 36 persons who experienced oral allergy syndrome after the ingestion of fruits in the family Rosaceae. This study cohort was divided into two groups: 18 without allergy to birch pollen (patients) and 18 with birch pollen allergy (control subjects). All were tested by skin prick tests (SPTs) with fresh Golden Delicious apple, fresh peach, and extracts of peel and pulp from both fruits. Their specific IgE reactivities against peach peel extract were further investigated by immunoblot analysis.

Results: All 18 subjects in the control group showed strongly positive skin reactions with both fresh apple and fresh peach, whereas no skin reactivity was found with extracts from peach peel, peach pulp, or apple pulp. Extract of apple peel produced positive skin reactions in 17 of 18 control subjects; however, the wheals were generally smaller than those induced by fresh fruits. Immunoblot analysis showed no reactivity for peach peel extract. In contrast, the SPTs with fresh fruits showed that some of the 18 patients had strongly positive reactions, but others had weak reactions or negative responses. Further, in a high proportion of the patients, consecutive SPT with fresh apple yielded inconsistent results. In all patients, SPTs with extracts from apple pulp and peach pulp were negative, whereas SPTs with peel extracts were strongly positive in all patients. In most patients, the wheal area induced by SPT with peel extracts was larger than that induced by SPTs with fresh fruits. Immunoblot analysis showed that serum specimens from all 18 patients reacted with a 10-kD protein in peach peel. This is the molecular mass of LTPs.

Conclusions: In birch pollen-allergic patients, the SPTs with fresh foods still remains the most reliable method of diagnosing vegetable food hypersensitivity. In contrast, in patients not allergic to birch pollen, the most reliable strategy for detection of patients sensitive to LTPs is skin prick testing with properly prepared fruit peel extracts. The loss of Bet v 1- and Bet v 2-like structures, which probably occurs during extraction, may facilitate immediate identification of the relevant allergen.

Ann Allergy Asthma Immunol 2001;87:68–71.

INTRODUCTION

Lipid transfer proteins (LTPs) are 10-kD polypeptides that have been de-

tected in many plant-derived foods.^{1–11}

In patients who are allergic to Rosaceae (apple, peach, apricot, cherry, plum, and pear) but not allergic to birch pollen, recent studies have shown that LTPs are the major allergen.^{12–16} In patients who are allergic to both Rosaceae and birch pollen (attributable to cross-reactivity with birch allergens, such as Bet v 1 and Bet v 2),

the allergic reaction is characterized by the oral allergy syndrome (OAS). In contrast, most patients sensitive to LTPs frequently experience both the OAS and systemic symptoms after eating an offending food,¹⁷ and some reports of life-threatening anaphylaxis have been published.^{18,19} Thus, the detection of LTP-reactive patients may be clinically useful.

At present, LTP-sensitive patients can be identified only by immunoblot analysis, a method that is time-consuming, expensive, and not available in most allergy departments. Recombinant LTPs, as well as LTPs purified from single-vegetable foods, are not yet available for routine skin prick tests (SPTs). In this study, our goal was to find a sensitive, specific, and easy method for detecting LTP-sensitive patients.

PATIENTS AND METHODS

Study Population

We studied 36 persons with typical OAS after the ingestion of fruits belonging to the family Rosaceae. Of this overall study cohort, 18 (the patient group) did not have birch pollen sensitivity, as shown by the absence of seasonal respiratory symptoms and by negative results of SPTs with commercial birch pollen extract (Allergopharma, Reinbek, Germany). The other 18 (the control group) had birch pollinosis, as manifested by rhinoconjunctivitis with or without asthma from the end of February to the end of May and by strongly positive results of SPTs with birch pollen extract.

Skin Tests

Patients and control subjects underwent SPTs with both fresh Golden Delicious apples and fresh peaches by the prick + prick technique²⁰ and with ex-

* Ambulatorio di Allergologia, Ospedale Caduti Bollatesi, Bollate (MI), Italy.

† Lofarma SpA, Milan, Italy.

Received for publication February 7, 2000.

Accepted for publication in revised form January 31, 2001.

tracts of peel and pulp of both fruits prepared at Lofarma Laboratories (Milan, Italy). To evaluate the reproducibility of SPTs with fresh Golden Delicious apples, we tested 16 patients and all 18 control subjects by the same technique on at least two distinct occasions.

All SPTs were performed on the volar side of the forearm with standard disposable 1-mm-tip prick lancets (Dome/Hollister-Stier, Milan, Italy). After 15 minutes, measurements were made, and skin reactivities were graded by comparison with the positive control wheal induced by 10 mg/mL histamine. Wheals <2+ (ie, with diameters <50% of the diameter of the positive control) were considered negative.²¹ Wheals with diameters of at least 50%, 100%, and >100% of the diameter of the positive control were considered 2+, 3+, and 4+ skin reactivities, respectively.

Food Extract Preparation

The peel or pulp of Granny Smith apples and peaches was separately homogenized at 10% (wt/vol) in 0.1 mol/L potassium phosphate buffer, pH 7.0, containing 2% polyvinylpyrrolidone, 7 mmol/L diethyldithiocarbamate, 2 mmol/L ethylenediaminetetraacetic acid disodium salt, and 2.6 mmol/L NaN₃ to remove phenolic substances that could lead to partial denaturation of certain allergenic proteins.²² Particulate materials were removed by centrifugation at 18,000 *g* for 30 minutes. The supernatants of peel extracts were dialyzed against 5 mmol/L ammonium bicarbonate and, after lyophilization, resuspended in 1/10 (apple) or 1/5 (peach) of the initial volume with saline containing 0.4% phenol. The supernatants of the pulp extracts were dialyzed directly against saline because the large amounts of pectin did not allow lyophilization of the extracts. All extracts were finally diluted 1:2 with glycerol containing 0.4% phenol for preparation of the SPT solutions.

Immunoblot Analysis

The presence of IgE antibodies specific for peach peel proteins was also

Table 1. Skin Test Results in Rosaceae-Sensitive and Birch Pollen-Sensitive Control Subjects*

Control Subject (no.)	Apple				Peach		
	Fresh 1	Fresh 2	Peel	Pulp	Fresh	Peel	Pulp
1	4+	4+	2+	Neg	3+	Neg	Neg
2	4+	4+	2+	Neg	3+	Neg	Neg
3	3+	4+	3+	Neg	4+	Neg	Neg
4	4+	4+	2+	Neg	4+	Neg	Neg
5	4+	3+	3+	Neg	3+	Neg	Neg
6	3+	3+	2+	Neg	3+	Neg	Neg
7	3+	3+	Neg	Neg	2+	Neg	Neg
8	3+	3+	2+	Neg	3+	Neg	Neg
9	4+	4+	3+	Neg	3+	Neg	Neg
10	3+	4+	3+	Neg	4+	Neg	Neg
11	4+	3+	3+	Neg	3+	Neg	Neg
12	3+	3+	2+	Neg	3+	Neg	Neg
13	4+	4+	4+	Neg	2+	Neg	Neg
14	3+	4+	4+	Neg	3+	Neg	Neg
15	4+	4+	3+	Neg	3+	Neg	Neg
16	4+	3+	2+	Neg	2+	Neg	Neg
17	4+	3+	2+	Neg	3+	Neg	Neg
18	3+	4+	3+	Neg	3+	Neg	Neg

* Fresh, SPTs with fresh fruit; Fresh 1 and 2, consecutive SPTs with fresh apple; Neg, negative; Peel and Pulp, SPTs with fruit extracts.

evaluated by immunoblot analysis in serum specimens from every study participant. The same peel extract used to perform SPTs was also used for the immunoblot analysis. Electrophoresis of samples was done with 10% polyacrylamide precast gels (Nupage Bis-Tris; Novex, Prodotti Gianni, Milan, Italy) at 180 mA for 1 hour. The resolved proteins were transferred for 1

hour onto a nitrocellulose membrane, in accordance with the method of Towbin et al.²³ The membrane was saturated with 0.1 mol/L Tris-buffered saline containing 5% fat-free milk powder and incubated with sera for 16 hours at 4° C. After three washings, bound specific IgE was detected by peroxidase-conjugated anti-human IgE from goat (1:1,000 in saturation buffer;

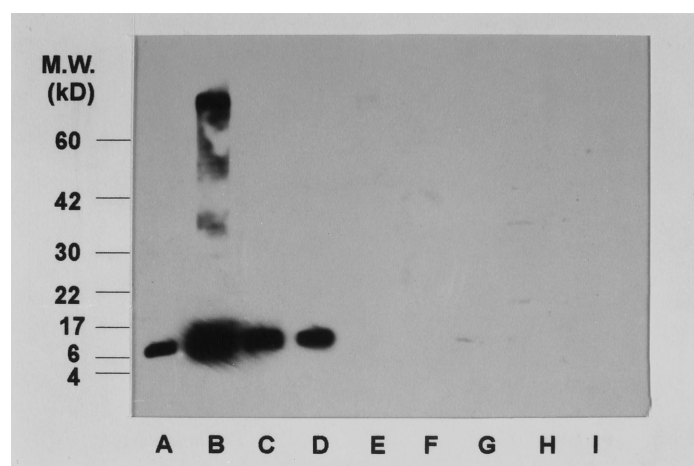


Figure 1. Immunoblot analysis of IgE antibodies for allergens in peach peel extract. Lanes A through D, Test results of serum specimens from patients allergic to Rosaceae but not allergic to birch pollen. Lanes E through H, Test results of serum specimens from control subjects allergic to both Rosaceae and birch pollen. Lane I, Test result of serum from a nonallergic normal control subject. M.W., molecular weight.

KPL Cellbio, Milan, Italy) with an ECL Western blotting kit (Amersham, Milan, Italy) as substrate.

RESULTS

Skin Test Results in Control Group

The SPT results for subjects in the control group who experienced OAS after eating fruits in the family Rosaceae and who were also allergic to birch pollen are shown in Table 1. All control subjects showed strongly positive skin reactions with both fresh apples and fresh peaches. Consecutive tests with fresh apples showed no appreciable variation of skin reactivity (ie, of diagnostic sensitivity) throughout time. No reactivity was found after SPTs with peach peel, peach pulp, or apple pulp extracts. In contrast, SPTs with apple peel extract was positive in 17 of 18 control subjects; however, wheals were usually smaller than those induced by the fresh fruits (Table 1). On immunoblot analysis of serum samples from four control subjects, no reactivity was observed against peach peel extract (Fig 1).

Skin Test Results in Patient Group

The SPT results for the patients who experienced OAS after eating fruits in

the family Rosaceae but who were not allergic to birch pollen are shown in Table 2. The results with fresh fruits were variable. Some patients showed strongly positive skin reactions, whereas other skin reactions were weak or even negative. Surprisingly, the reproducibility of SPTs with fresh apples was poor; in 9 of 16 patients, the consecutive skin tests showed both positive and negative results. The SPT results with both apple pulp and peach pulp extracts were negative in all patients. In contrast, both peel extracts elicited strongly positive skin responses in all patients. In most patients, the area of the wheal induced by peel extracts was substantially larger than that induced by the corresponding fresh fruit extracts.

In studies of peach peel extracts, immunoblot analysis of serum specimens from four patients showed reactions in all, with a band at 10 kD. This molecular mass corresponds to that of LTPs. One patient also showed IgE antibodies to higher molecular mass proteins up to 90 kD. In other immunoblot experiments (results not shown), sera from all 18 patients reacted with peach peel extract.

DISCUSSION

In patients with vegetable food allergy, the poor sensitivity of SPTs with commercial extracts is mainly attributable to the loss of relevant allergens.²² A previous study showed that disruption of the fruit rapidly degrades the Bet v 1 homologous structures.²⁴ This result was fully confirmed in the birch pollen- and Rosaceae-sensitive control subjects included in the current study. These control subjects showed strong skin responses after SPTs with fresh apples and fresh peaches but totally negative responses after SPTs with both peach and apple pulp extracts. The apple peel extract retained some Bet v 1-like activity, as previously observed.²⁵ The loss of Bet v 1 homologous structures, which probably occurs during extraction procedures, was confirmed by the absence of IgE antibodies in control serum specimens against peach peel extract on immunoblot analysis. In birch pollen-allergic patients, the SPTs with fresh foods by the prick + prick technique remains the most reliable method of diagnosing vegetable food hypersensitivity.^{20,26}

Patients sensitive to LTP showed totally different responses to in vivo diagnostic tests. These patients strongly reacted after SPTs to both apple and peach peel extracts but showed no reactivity against both pulp extracts. Further, SPTs with fresh apples and peaches by the prick + prick technique induced variable and poorly reproducible results. At least two important facts may explain these results. First, LTPs are stable allergens that are not destroyed by extraction procedures; this fact is clearly shown by the immunoblot analysis. Second, LTPs are mainly present in the most superficial layers of vegetable foods.^{1,7,12} From a clinical standpoint, this explains why several LTP-sensitive patients tolerate the offending fruits if they are peeled. From a diagnostic point of view, this explains why most commercial extracts contain negligible amounts of LTPs; they have probably been prepared with an excess of pulp over peel. With the prick + prick technique, the standard lancet is pushed through the fruit peel

Table 2. Skin Test Results in Rosaceae-Sensitive Patients With No Sensitivity to Birch Pollen*

Patient (no.)	Apple				Peach		
	Fresh 1	Fresh 2	Peel	Pulp	Fresh	Peel	Pulp
1	Neg	4+	4+	Neg	Neg	3+	Neg
2	3+	Neg	4+	Neg	4+	4+	Neg
3	4+	Neg	4+	Neg	Neg	4+	Neg
4	Neg	3+	4+	Neg	2+	4+	Neg
5	4+	3+	4+	Neg	4+	4+	Neg
6	Neg	Neg	3+	Neg	2+	4+	Neg
7	Neg	2+	4+	Neg	2+	4+	Neg
8	4+	2+	4+	Neg	3+	4+	Neg
9	2+	3+	3+	Neg	3+	4+	Neg
10	3+	Neg	3+	Neg	4+	4+	Neg
11	Neg	Neg	3+	Neg	4+	4+	Neg
12	Neg	Neg	3+	Neg	3+	4+	Neg
13	Neg	Neg	3+	Neg	Neg	3+	Neg
14	Neg	3+	4+	Neg	2+	3+	Neg
15	Neg	2+	3+	Neg	3+	4+	Neg
16	2+	Neg	4+	Neg	3+	2+	Neg
17	2+	ND	4+	Neg	Neg	3+	Neg
18	3+	ND	4+	Neg	4+	3+	Neg

* ND, not done. For other explanations, see Table 1.

and dipped deeply into the pulp; most likely, this action causes a variable (ranging from negligible to total) loss of LTPs present on the tip of the lancet and explains why SPT results with fresh fruits are often inconsistent in these patients.

CONCLUSION

SPTs with properly prepared fruit peel extracts rather than SPTs with fresh fruits seem the most reliable and easiest way to detect Rosaceae-allergic patients who are sensitive to LTPs. In these fruit peel extracts, the total loss of Bet v 1- and Bet v 2-like allergenic activity during extraction allows an easy and immediate identification of the allergenic proteins responsible for allergic symptoms.

REFERENCES

- Garcia-Olmedo F, Molina A, Segura A, Moreno M. The defensive role of non-specific lipid-transfer proteins in plants. *Trends Microbiol* 1995;3:72–74.
- Molina A, Segura A, Garcia-Olmedo F. Lipid transfer proteins (nsLTPs) from barley and maize leaves are potent inhibitors of bacterial and fungal plant pathogens. *FEBS Lett* 1993;316:119–122.
- Cammue BP, Thevissen K, Hendriks M, et al. A potent antimicrobial protein from onion seeds showing sequence homology to plant lipid transfer proteins. *Plant Physiol* 1995;109:445–455.
- Torres-Schumann S, Godoy JA, Pin-tor-Toro JA. A probable lipid transfer protein gene is induced by NaCl in stems of tomato plants. *Plant Mol Biol* 1992;18:749–757.
- Ostergaard J, Vergnolle C, Schoentgen F, Kader JC. Acyl-binding/lipid-transfer proteins from rape seedlings, a novel category of proteins interacting with lipids. *Biochim Biophys Acta* 1993;1170:109–117.
- Pelese-Siebenbourg F, Caelles C, Kader JC, et al. A pair of genes coding for lipid-transfer proteins in *Sorghum vulgare*. *Gene* 1994;148:305–308.
- Pyee J, Yu H, Kolattukudy PE. Identification of a lipid transfer protein as the major protein in the surface wax of broccoli (*Brassica oleracea*) leaves. *Arch Biochem Biophys* 1994;311:460–468.
- Meijer EA, de Vries SC, Sterk P, et al. Characterization of the non-specific lipid transfer protein EP2 from carrot (*Daucus carota* L.). *Mol Cell Biochem* 1993;123:159–166.
- Vignols F, Lund G, Pammi S, et al. Characterization of a rice gene coding for a lipid transfer protein. *Gene* 1994;142:265–270.
- Coutos-Thevenot P, Jouenne T, Maes O, et al. Four 9-kDa proteins excreted by somatic embryos of grapevine are isoforms of lipid transfer proteins. *Eur J Biochem* 1993;217:885–889.
- Aronel V, Kader JC. Lipid transfer in plants. *Experientia* 1990;46:579–585.
- Lleonart R, Cistero A, Carreira J, et al. Food allergy: identification of the major IgE-binding component of the peach (*Prunus persica*). *Ann Allergy* 1992;69:128–130.
- Pastorello EA, Ortolani C, Farioli L, et al. Allergenic cross-reactivity among peach, apricot, plum, and cherry in patients with oral allergy syndrome: an in vivo and in vitro study. *J Allergy Clin Immunol* 1994;94:699–707.
- Pastorello EA, Farioli L, Pravettoni V, et al. The major allergen of peach (*Prunus persica*) is a lipid transfer protein. *J Allergy Clin Immunol* 1999;103:520–526.
- Pastorello EA, Pravettoni V, Farioli L, et al. Clinical role of a lipid transfer protein that acts as a new apple-specific allergen. *J Allergy Clin Immunol* 1999;104:1099–1106.
- Fernandez-Rivas M, van Ree R, Cuevas M. Allergy to Rosaceae fruits without related pollinosis. *J Allergy Clin Immunol* 1997;100:728–733.
- Asero R. Detection and clinical characterization of patients with oral allergy syndrome caused by stable allergens in Rosaceae and nuts. *Ann Allergy Asthma Immunol* 1999;83:377–383.
- Navarro AM, Orta JC, Sanchez MC, et al. Primary sensitization to *Morus alba*. *Allergy* 1997;52:1144–1145.
- Escribano MM, Munoz FJ, Velazquez E, et al. Anaphylactic reaction caused by cherry ingestion. *Allergy* 1996;51:756–757.
- Ortolani C, Ispano M, Pastorello EA, et al. Comparison of results of skin prick tests (with fresh foods and commercial food extracts) and RAST in 100 patients with oral allergy syndrome. *J Allergy Clin Immunol* 1989;83:683–690.
- Bousquet J, Michel FB. In vivo methods for the study of allergy: skin tests, techniques and interpretation. In: Middleton E, Reed CE, Ellis EF, et al, eds. *Allergy: Principles and Practice*. 4th ed. St. Louis, MO: Mosby, 1993:573–594.
- Bjorksten F, Halmepuro L, Hannuksela M, et al. Extraction and properties of apple allergens. *Allergy* 1980;35:671–677.
- Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979;76:4350–4354.
- Rudeschko O, Fahlbusch B, Henzgen M, et al. Investigation of the stability of apple allergen extracts. *Allergy* 1995;50:575–580.
- Asero R, Mistrello G, Roncarolo D, et al. A new apple extract. *Allergy* 1999;54:87–88.
- Lahti A, Hannuksela M. Hypersensitivity to apple and carrot can be reliably detected with fresh material. *Allergy* 1978;33:143–146.

Requests for reprints should be addressed to:
Dr. Riccardo Asero
Ambulatorio di Allergologia
Ospedale Caduti Bollatesi
Via Piave 20
20021 Bollate (MI), Italy
E-mail: r.asero@libero.it