

Original article

Double-blind, placebo-controlled food challenge with apple

The aim of the study was to develop and evaluate different methods of double-blind, placebo-controlled food challenge (DBPCFC) with apple. Three different DBPCFC models were evaluated: fresh apple juice, freshly grated apple, and freeze-dried apple powder. All challenges were performed outside the pollen season and took place from 1997 to 1999. The freeze-dried apple material was characterized by means of leukocyte histamine release (HR), skin prick test (SPT), and immunoblotting experiments. The study population consisted of birch pollen-allergic patients with a history of rhinitis in the birch-pollen season and positive specific IgE to birch. For comparison of the DBPCFC models, 65 patients with a positive open oral challenge with apple were selected. In the characterization of the freeze-dried apple material, 46 birch pollen-allergic patients were included. The IgE reactivity to apple was evaluated by measurement of specific IgE, HR, and SPT. Golden Delicious apples were used in all experiments. The results of this study showed that it was possible to perform DBPCFC with apple in birch pollen-allergic individuals. The model with freshly squeezed apple juice had a low sensitivity and displayed a high frequency of reactions to placebo, probably due to the ingredients used for blinding. The sensitivity of the models with freshly grated apple and freeze-dried apple powder was 0.74/0.60. An increase in sensitivity is desirable. The freeze-dried apple powder proved to be useful for SPT, HR, and oral challenges, but further investigation of the stability and the allergenic profile of the material is needed.

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Key words: apple; birch; cross-reaction; DBPCFC; food allergy; lyophilization; tachyphylaxis.

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Accepted for publication 7 September 2000

The cross-reactivity between birch pollen and various fruits, nuts, and vegetables has been intensively investigated over the last 25 years (1–6). Isolation, characterization, and cloning of major allergens from different plant foods, such as Mal d 1 from apple, have revealed a high degree of sequential homology with Bet v 1 (7–9). Different serologic and clinical aspects of the cross-reactions between birch pollen and apple have been investigated in numerous studies, but only a subgroup have included food challenges (5, 6, 10–19); of these, even fewer have evaluated patient groups of 10 or more (5, 10, 12, 17–19). Food challenge with apple may be difficult to perform, since apple allergens seem very susceptible to all types of processing of the fruit (14, 20). Moreover, the allergenicity differs between apple strains and is influenced by ripening and storage of the fruits (14, 21, 22). As opposed to classical food allergy, such as that to milk, egg, or fish, where the final diagnosis is based on the outcome of double-blind, placebo-controlled food challenges (DBPCFC) (23), open challenges are generally considered confirmatory for allergenic cross-reactions between pollen and foods. A history of allergic rhinitis in the birch-pollen season combined with the detection of

specific IgE to birch increases the credibility of suspected reactions to various plant foods, but there is still a need for confirmation of the diagnosis of food allergy in some cases. Both in the investigation of severe food allergic reactions and for scientific use, a consistent DBPCFC model would be valuable.

The aim of the study was to develop and evaluate different methods of DBPCFC with apple.

Material and methods

Design

The study compared three different models of blinded challenge with apple. All challenges were performed outside the pollen season, and took place from 1997 to 1999 at the Allergy Unit at the National University Hospital in Copenhagen (Denmark). The clinical reactivity to apple was assessed by open and blinded food challenges with apple. Freshly squeezed apple juice (model I), freshly grated apple (model II), and freeze-dried apple powder (model III) were used in the DBPCFC. IgE reactivity to apple was evaluated by measurement of specific IgE and HR, and by SPT. Furthermore, the freeze-dried material was characterized by means of HR, SPT, and immunoblotting experiments. French Golden Delicious (GD) apples bought at the local market were used in all experiments.

The study was approved by the local ethics committee ([KFJ]02–156/96 and 01–075/97), and all subjects gave written informed consent before entering the study.

Study population

The study population consisted of birch pollen-allergic patients with a history of rhinitis in the birch-pollen season and positive specific IgE to birch. For comparison of the DBPCFC models, patients with a positive open oral challenge with apple were selected. Results from 65 patients were evaluated: 40, 19, and 25 for models I, II, and III, respectively.

For the characterization of the freeze-dried apple material, a total of 46 birch pollen-allergic patients (positive open challenge with apple $n=28$ /negative challenge $n=18$) were included. Each procedure used was tested in overlapping subgroups of patients.

Medication

Before challenges and SPT, medication was discontinued according to the guidelines on skin testing of the European Academy of Allergology and Clinical Immunology (EAACI) (24). Sixteen patients in group III had received birch-pollen immunotherapy and one patient used inhaled corticosteroids.

Preparation of freeze-dried apple

Fresh GD apples were washed, the cores and stalks were removed, and the apples were cut into small pieces (about 1×1 cm) and immediately immersed in a large glass container with liquid nitrogen. The frozen apple pieces and remaining nitrogen were transferred to disposable aluminum trays (one layer of apple in the trays) and placed on shelves in an external acrylic chamber of a freeze-dryer (Alpha 1–4, Crist, Osterode am Harz, Germany). An amount of 844 g (800–918 g) of fresh apple was processed per drying session. The mean lyophilization time was 46 h (35–54). The following presetting values were chosen for the main drying process: vacuum pressure 1 mbar (0.52–1.03), drying temperature -15°C , and safety pressure 1.65 mbar. The pressure was reduced during the last part of the freeze-drying process in order to remove the remaining water. The freeze-dried material was stored at -80°C . For oral challenges, HR, and SPT, the freeze-dried apple material was pulverized for 3×10 s in a food processor (Braun, Kronberg, Germany) and immediately transferred to small plastic containers. During weighing and transferring, the material was kept on ice.

Extracts of freeze-dried apple for SPT and HR

The freeze-dried material used for both SPT and HR was extracted in isotonic saline in a ratio of 1:3 or 1:6 (w/v). Material exclusively for HR was extracted in Pipes-AMC (pH 7.4) (10 mM piperazine-*N,N*-[bis-ethane sulfonic acid], 140 mM sodium acetate, 5 mM potassium acetate, 0.6 mM CaCl_2 , 1.1 mM MgCl_2 , glucose 1 mg/ml, human serum albumin 0.3 mg/ml, heparin [Leo, Ballerup, Denmark] 15 IU/ml) in a ratio of 1:12 (w/v).

Material from six drying sessions was compared, and different parameters in the extraction procedure were evaluated by HR: blending, additional extraction time (30 min), and centrifugation (1570 g; 2×10 min). In addition, the stability of the extracts was evaluated by HR and SPT, comparing the effect of storage time (1–96 h), storage temperature ($4^\circ\text{C}/21^\circ\text{C}$), and stabilization with glycerol 87% (1:1 v/v) after extraction. All comparisons were performed on extracts of equal weight ratios.

SPT

SPT was performed according to EAACI guidelines (24). All patients were tested with birch-pollen extract (Soluprick, ALK-Abelló,

Hørsholm, Denmark) and fresh GD apples. SPT with fresh apples was performed by the prick-prick technique (2, 5). The skin wheal areas were determined by computer scanning (25).

Leukocyte HR

The HR experiment was performed by the glass-fiber method (26). Fresh GD and freeze-dried apple material were used for HR. Fresh apple was prepared as follows. An amount of 10 g fresh apple was crunched in a Stomacher 80 (Seward Lab System, UK) at high speed for 120 s with 10 ml Pipes-AMC. After centrifugation (2000 g; 10 min), the supernatant was used as stock solution. The fresh preparation was applied within 15 min. For technical reasons, the extract of freeze-dried apple stabilized in glycerol and the comparable extract without glycerol were diluted 10 times with Pipes buffer. All extracts were added to the plates in nine 3.5-fold dilutions in Pipes buffer.

An HR test was considered positive when the maximal release was ≥ 14 ng/ml. The HR was calculated as the mean value of the maximum release (ng/ml) and as the number of 3.5-fold dilutions of the apple extract at which the release was 50% of the maximal release (50% maximum HR); e.g., the higher the number, the higher the allergenic potency of the extract.

Specific IgE

Specific IgE against apple and birch pollen was measured by the CAP-System (Pharmacia-Upjohn, Uppsala, Sweden), according to the instructions of the manufacturer.

Immunoblotting

SDS-PAGE, by the method of Laemmli (27), and immunoblotting experiments were conducted with the freeze-dried apple powder as described for apple extract (7, 14), with the exception that streptavidin peroxidase was replaced with streptavidin alkaline phosphatase. The apple powder was extracted in distilled water at a ratio of 1:2.5 (w/v). For SDS-PAGE, a 13% acrylamide separation gel and a 5% stacking gel were prepared (Rotiphorese Gel30, Roth, Karlsruhe, Germany), and 85 μg of protein was loaded on the gel (15 μg protein per cm). The proteins were transferred to a nitrocellulose (NC) membrane, and, after electroblotting and blocking, NC strips were incubated with either serum or antibody. Three sera from challenge-positive patients were selected. A serum without specific IgE against apple was used as negative control. Monoclonal mouse antibodies against Bet v 1 (Paul-Ehrlich Institut, Langen, Germany) and Mal d 1 (Dr B. Fahlbusch, University of Jena, Germany [8]) were used, and a non-sense antibody against pig-gamma-globulin (Paul-Ehrlich Institut, Langen, Germany) was used as negative control.

Oral challenges

GD apples were used for all three challenge models. The commercial fruit juices, apple juice (1:5 v/v) in models II and III, and blackcurrant juice (1:5 v/v) in models I and III, were pasteurized products.

Model I: fresh apple juice

Stalks and cores were removed, and the apple pieces were transferred to a juice extractor (Braun, Kronberg, Germany). One apple of 140–150 g produced approximately 100 ml of juice.

The recipe and the dosage regimen were optimized during the study. The total volume of apple juice in a challenge varied between 50 and 95 ml, and the maximum volume of apple juice in a dose was between 20 and 50 ml. Moreover, the ratio of apple juice to placebo

mixture in a dose changed from 1:4 to 1:0.6 v/v. To blind the greater volume of apple juice, additional blackcurrant juice were used for the active challenges.

Final recipe:	
Placebo mixture:	120 ml concentrated blackcurrant juice
	140 ml tap water
	40 g Elemental 028, neutral (SHS, Manchester, UK)

Placebo challenge	Active challenge			
	Placebo	Placebo	Blackcurrant juice	Fresh apple juice
0	25 ml	25 ml	–	–
1	25 ml	20 ml	–	5 ml
2	100 ml	50 ml	10 ml	40 ml
3	80 ml	10 ml	20 ml	50 ml

Each dose was served in a cup with a lid and straw.

Model II: freshly grated apple

Placebo challenge	Active challenge
22.5 g finely grated cabbage	17.0 g finely grated cabbage
7.5 ml concentrated apple juice	3.0 ml concentrated apple juice
–	20.0 g finely grated apple

The challenge was served inside a little pita bread (approx. 30.0 g).

Model III: freeze-dried apple powder

Placebo mixture:	40 g boiled and mashed potato
	10 g grated and blanched cabbage
	20 ml concentrated apple juice

Placebo challenge	Active challenge
32 g placebo mixture	25 g placebo mixture
–	3 or 4 g freeze-dried apple powder
1 ml concentrated apple juice	4 ml concentrated apple juice
1 ml concentrated blackcurrant juice	1 ml concentrated blackcurrant juice

Each challenge was served on a plate and was eaten with a spoon.

All active challenges were served within a few minutes after preparation.

For model I, the active and the placebo challenges were performed on different days (>48 h between the challenges). In models II and III, both the active and the placebo challenges were served to the patient on the same day. The first dose at each challenge session was a test dose without apple (placebo) served single-blindedly. In model III, the first active dose was 3 g of apple powder; if there were no symptoms or very weak symptoms, an additional single-blinded dose of 4 g of apple powder was added. In model I, the challenge days were randomized; in models II and III, the challenge order on each challenge day was randomized. The challenges were prepared and randomized by a dietitian and administered double-blindedly to the patients. The code was not broken until the challenge procedure was completed, and the results were entered in an observation sheet. The interval between the challenge doses was a minimum of 20–30 min. The next dose or challenge was not served before symptoms had disappeared or diminished significantly. The challenge session (in model I, the second challenge day) was concluded with an open challenge test with fresh apple. A slice (about 10 g) of apple was administered to the patients. If this amount of fresh apple was tolerated, the patient was allowed to continue to eat up to one apple, bite for bite.

The patients were under continuous observation during the challenge tests, and possible symptoms were recorded. The severity of the reactions to the individual challenges was evaluated by the patient and graded on a scale from 0 to 3, a score of 3 indicating the most pronounced symptoms.

Statistics

The SPT, HR, and IgE results of the patient groups were compared by parametric (*t*-test) or nonparametric methods (Mann–Whitney rank sum test) according to normality and the equal variance test suggested by the software. The symptom scores were compared by Mann–Whitney rank sum test. The sensitivity of the DBPCFC models was calculated according to Bindslev-Jensen & Poulsen (28). Disease was defined as a positive clinical history of allergy to birch pollen and positive specific IgE to birch, combined with a positive open challenge test with apples. Sensitivity, placebo reactions, and difference from random sample were compared by the chi-square test. The freeze-dried material was compared by Wilcoxon signed rank test, except for the comparison of material from different drying sessions (Kruskal-Wallis one-way analysis of variance on ranks). The programs used were SigmaStat 1.0 (Jandel Corporation, San Rafael, CA, USA) and Statistica 4.5 (StatSoft, Inc., Tulsa, OK, USA).

Results

Table 1 shows the results of the open challenge, SPT, and HR and the level of specific IgE of the three patient groups. Group I had significantly higher symptom scores upon open challenge with fresh apple than group III ($P < 0.001$). No differences were seen between groups I and II or II and III. Groups I and II had higher skin wheal area to birch than group III ($P = 0.02/0.04$). The maximum HR (ng/ml) against fresh GD apple was higher in group III than in group I ($P = 0.02$). No difference was found in 50% maximum HR between groups III and group I. There were no significant differences in serum specific IgE to either apple or birch pollen between the groups.

Blinded challenges

The outcome of the blinded challenges is described in Table 2. Exactly 17, 14, and 15 blinded challenges were true-positive (positive reaction to active and concomitant negative reaction to placebo and the test dose), corresponding to a sensitivity of 0.43 (17/40), 0.74 (14/19), and 0.60 (15/25) for models I, II, and III, respectively. Models II and III had a significantly higher sensitivity than model I ($P < 0.00001/P = 0.02$). The numbers of observed reactions to placebo were 10 (model I), 0 (model II), and 4 (model III). The frequency of placebo reactions was lower in model II than in both models I ($P < 0.00001$) and III ($P = 0.0001$). Models I and III did not differ significantly. The median scores of the blinded models were not significantly different. Table 2 also shows the sensitivity of the models if the placebo reactors were excluded from the material. The distribution between reactions to active and placebo was significantly different from the random sample in models II (14/0,

Table 1. Open oral challenge, SPT, HR, and specific IgE

		Model I (<i>n</i> = 40)	Model II (<i>n</i> = 19)	Model III (<i>n</i> = 25)	Difference (<i>P</i>)		
					I–I	I–II	I–III
Open challenge							
Fresh GD	Symptom score (median [range])	2 (1–2)	2 (1–2)	2 (1–2)	NS	< 0.001	NS
Skin prick test							
Fresh GD	Area in mm ² (mean [range])	23.1 (1.9–56.8)	30.9 (6.6–69.1)	27.1 (2.2–86.0)	NS	NS	NS
Birch	Area in mm ² (mean [range])	48.3 (1.7–174.6)	40.4 (9–75.2)	34.2 (0.7–148.1)	NS	0.02	0.04
Histamine release							
Fresh GD	Max. in ng/ml (mean [range])	33.1 (0–85)	47.3 (0–112)	51.5 (0–130)	NS	0.02	NS
Fresh GD	50% max. as no dilution (median [range])	5 (0–9)	6.5 (0–9)	6 (0–9)	NS	NS	NS
Specific IgE							
Apple	kU/l (mean [range])	4.9 (0–33.3)	4.9 (0–25.1)	2.4 (0–7.4)	NS	NS	NS
Birch	kU/l (mean [range])	44.9 (1.9–388)	29.4 (2.7–86)	29.7 (6.2–86.9)	NS	NS	NS

$P < 0.00001$) and III (15/4, $P = 0.004$), but not in model I (17/10). Due to practicable advantages, the freeze-dried material was subjected to further investigation.

Characterization of the freeze-dried material

The weight ratio of freeze-dried material to fresh apple was 1:7 (1:5.8–1:7.1). Freeze-dried material from six different drying sessions was compared, and there were no significant differences evaluated by HR in four patients. The effect of different extraction procedures was evaluated in parallel in the same patients. The numbers in parentheses indicate the number of patients included in the testing of each parameter. Blending of the freeze-dried apple material before extraction ($n = 32$) increased 50% maximum HR ($P = 0.02$), but maximum HR was unchanged. The process of centrifugation (1570 *g*; 2×10 min) ($n = 9$) after crunching gave a significantly higher 50% maximum HR ($P = 0.02$) than just crunching, but no difference was seen in maximal release. An additional 30 min of extraction time ($n = 9$) before centrifugation did not increase the activity.

The effect of storage time, storage temperature, and stabilization in glycerol evaluated by SPT and HR ($n = 4$) is illustrated in Figs. 1 and 2. Fig. 1 shows the effect of

storage for 96 h at 4°C and the effect of glycerol, evaluated with parallel HR and SPT data in four patients. The extracts (1:6 w/v in glycerol/isotonic saline or saline, 300 µg protein/ml) were used undiluted for SPT and after dilution $\times 10$ (~1:60 w/v) in Pipes buffer for HR. Maximum HR was unchanged after 24 h for the extract with glycerol (Fig. 1A), while the extract without glycerol (Fig. 1B) showed a decrease in maximum HR in two patients. After 96 h, the extract without glycerol showed a decrease in maximum HR for all four patients, compared to an unchanged activity of the extract with glycerol in two patients and a decrease in two. Evaluated by SPT, the activity of both extracts was unchanged after 96 h. There were no significant differences in skin wheal areas between the two extracts. Fig. 2 shows the HR dilution curves in four patients of extracts (1:3 w/v in isotonic saline, 580 µg protein/ml) stored at 4°C (Fig. 2A) and 21°C (Fig. 2B) for 96 h. The extract stored at 21°C gave no significant release in two patients after 96 h, while the extract stored at 4°C gave significant release in all four patients. A healthy nonallergic individual was used as control for SPT and HR, and no unspecific reactions were registered.

Immunoblotting (Fig. 3) revealed binding of all three

Table 2. Results of DBPCFC

	Model I (<i>n</i> = 40)	Model II (<i>n</i> = 19)	Model III (<i>n</i> = 25)	Difference (<i>P</i>)		
				I–I	I–II	I–III
DBPCFC						
Positive	17	14	15	–	–	–
Negative	13	5	6	–	–	–
Placebo reactions						
Number	10	0	4	<0.00001	NS	0.0001
Symptom score						
Median (range)	2 (1–2)	1 (1–2)	1 (1–3)	NS	NS	NS
Sensitivity						
Overall	0.43	0.74	0.60	NS	NS	NS
Excluding placebo reactors	0.57	0.74	0.71	0.02	NS	NS
Reaction to active-placebo						
Numbers	17–10	14–0	15–4	–	–	–
Difference (<i>P</i>) from random	NS	<0.00001	0.0008			

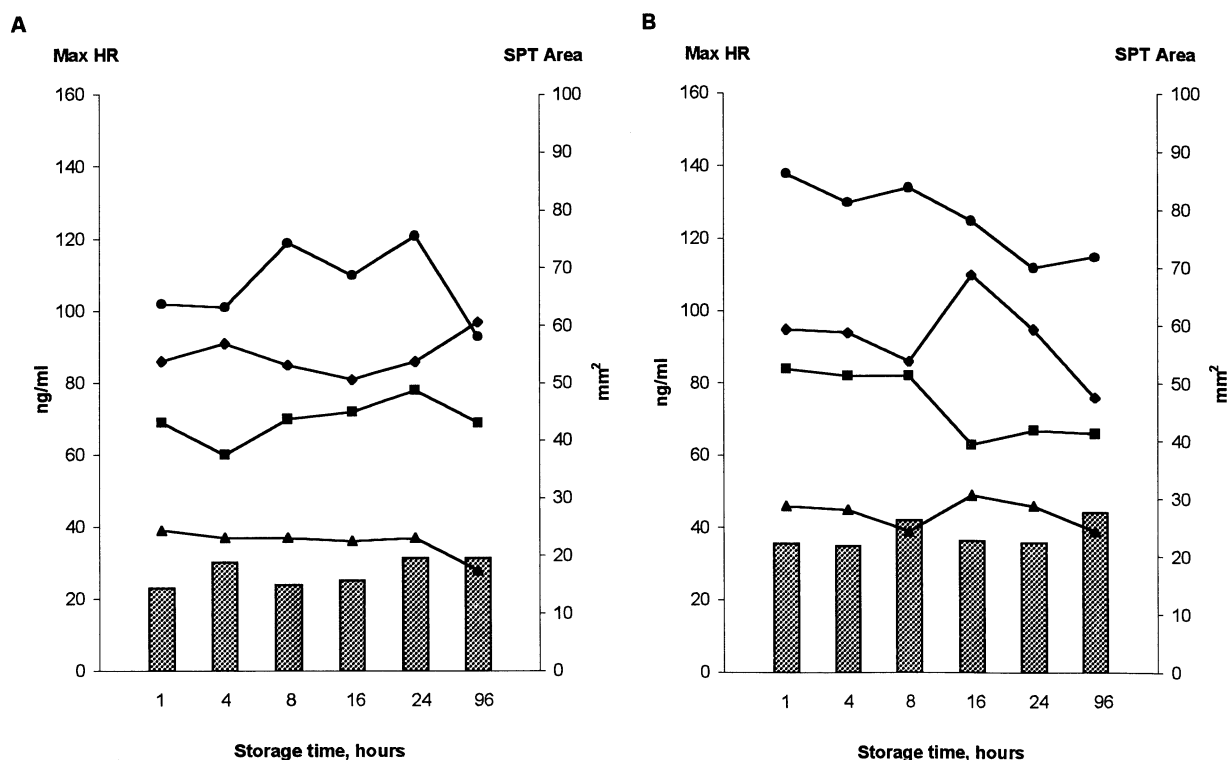


Figure 1. Stability of extracts with and without glycerol, evaluated by histamine release (curves) and skin prick test (bars) results, during storage for 96 h ($n=4$). Each curve represents one patient. Identical symbols in panels A and B and Fig. 2A and B correspond to same patients. SPT results are mean values for all four patients. A) Extract in 50% glycerol (~1:6 w/v). B) Extract in isotonic saline (~1:6 w/v). Extracts were used undiluted for SPT and diluted $\times 10$ in Pipes buffer for HR.

sera at a band with a molecular weight of approximately 18 kDa. No binding was seen for the nonallergic control. All strips, including the antibody marked, were developed for the same period of time, resulting in unspecific blackening of the antibody-marked strips. But the monoclonal antibody against Mal d 1 showed a pronounced binding at the 18-kDa band, while the Bet v 1-antibody gave a weaker but also significant binding. The non-sense antibody against pig-gamma-globulin did not lead to specific binding.

Discussion

In most studies that have included challenges with apple, the challenges have been performed openly, partly because apple allergens have been considered too unstable to process, and partly because a valid DBPCFC model was not available (5, 10, 12, 17–19). The issue of diagnostic accuracy has therefore not been addressed in these studies. A DBPCFC model for fresh fruit including apple has been published, but data on the efficacy of the model regarding allergy to apple were not specified (29). In a study on double-blind challenges with hazelnuts in 86 subjects, we found a sensitivity of 0.78 (30).

The challenge models evaluated in this study seem to differ both in sensitivity and number of placebo

reactions. The freshly grated apple (model II) had the highest sensitivity (0.74) and did not lead to unspecific reactions. This model has been used in a study on seasonal variation in allergy to apple (Skamstrup Hansen et al., submitted paper). In all, 52 blinded challenges were performed. Two reactions to placebo were recorded, one in a patient with no reaction to either active blinded challenge or open challenge with fresh apple. Model III (freeze-dried apple) had a comparable sensitivity (0.6) but significantly more placebo reactions, while the results of model I (freshly squeezed apple juice) with 17 reactions to active and 10 to placebo were indistinguishable from mere chance.

With a sensitivity of 0.6/0.74, the methods cannot detect the least sensitive patients. A larger amount or concentration of apple could probably increase the sensitivity, but apple has a characteristic smell and flavor and is therefore difficult to blind. We have no data on the stability of apple allergens in combination with the other ingredients of the active challenges in either of the models.

The selection of patients for this comparative study could pose a bias. The patients in models I and III were selected by history of allergy to birch pollen, and, depending on the result of the open challenge, included in the comparison of challenge models. Patients in model II were selected by history of allergy to birch pollen and

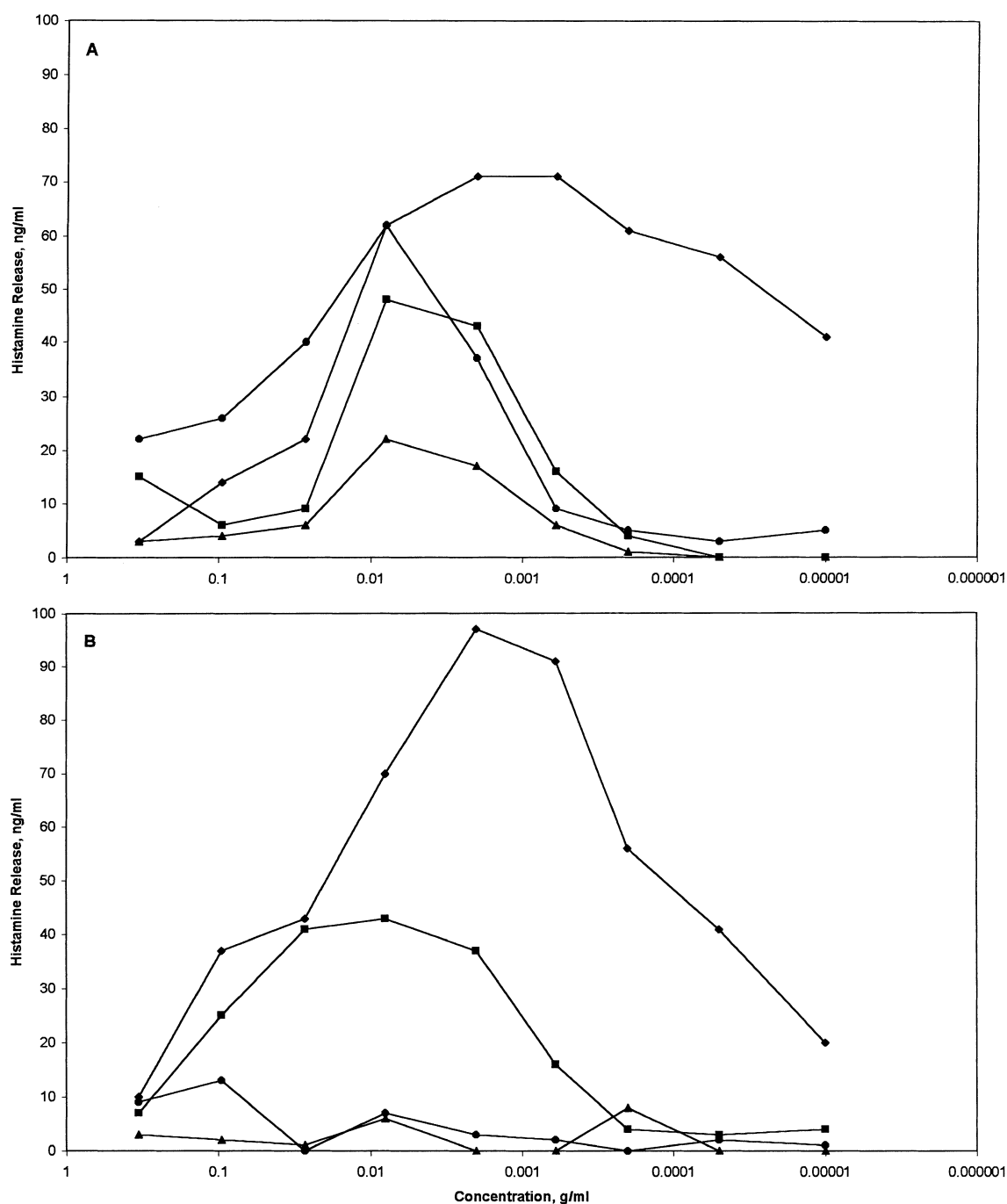


Figure 2. HR dilution curves after storage of extracts for 96 h at 4° and 21°C ($n=4$). Each curve represents one patient. Identical symbols in Figs. 1A and B and 2A and B correspond to same patients. The ordinate scale (concentration, g/ml) is log 10 and reversed, in order to indicate effect of dilution. A) Extract (1:3 w/v in isotonic saline) stored at 4°C. B) Extract (1:3 w/v in isotonic saline) stored at 21°C. No significant release for patients ▲ and ● with extract stored at 21°C.

concomitant food allergy to apples. They might therefore be more focused on this aspect of their allergic disease, more aware of symptoms, and perhaps also more clinically sensitive, even though they did not respond differently to fresh apple in the open challenge judged by the symptom scores. Moreover, the background data on open challenges indicate that the patients in group III were less clinically responsive than groups I and II. Some

of the patients in this group had received immunotherapy before this investigation. The importance of this selection bias is unknown.

The symptoms experienced by the patients in all three models were very mild, and most patients had OAS (oral allergy syndrome) (31) without any objective signs. A problem in all models could be the fact that the patients received more than one active challenge a day in the form

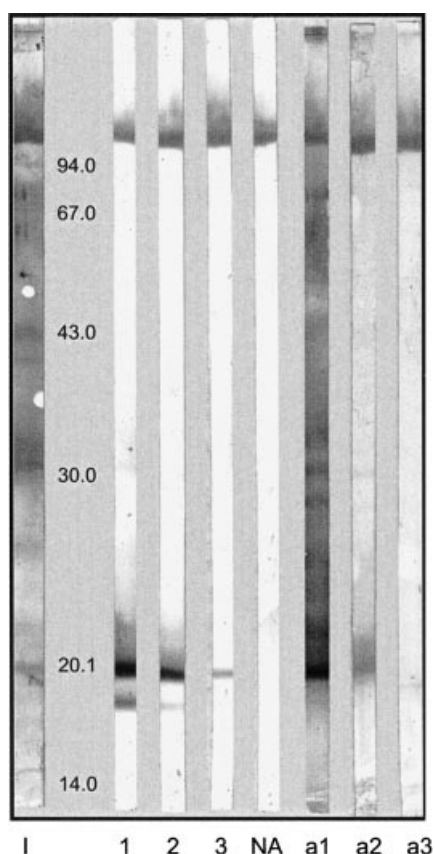


Figure 3. Immunodetection of extract of freeze-dried apple in three patient sera (no. 1–3), nonallergic control (NA) and three monoclonal antibodies (a1: mouse-a-Mal d 1; a2: mouse-a-Bet v 1; a3: mouse-a-pig-gamma-globulin). India ink colored NC strip (I). Molecular weight in kDa is indicated.

of more active doses or an active blinded challenge followed by an open challenge with fresh apple. In two patients included in model II, the open challenge was repeated on a separate day because of conflicting results. One of the patients had a mild reaction to the active blinded challenge but no reaction to the following open challenge. The second patient had no reaction upon either the blinded challenge or the open challenge in spite of a history of clinical symptoms and a positive open challenge performed earlier. Both challenges were positive. Development of tachyphylaxis is obviously a problem in interpretation of challenge results, determination of threshold doses, etc.

In model I, different regimens regarding doses and concentration of apple juice were tested (data not shown). Although the patient groups were too small for statistical analysis, increasing concentration seemed to be more important than increasing the amount – an observation that also could be ascribed to the development of tachyphylaxis during the challenge procedure. The liquid challenge of model I was included since it has proven to be useful for different food allergens (32, 33). The addition of concentrated blackcurrant juice to

disguise the taste of apple gave the mixture a very strong taste and produced a sensation in the mouth that could probably mimic OAS. Moreover, the use of commercial juices in a very high concentration could pose a problem, since it has been found that heat-stable IgE-binding lipid transfer proteins are present in commercial fruit juices (34). An Italian study showed that 28% of patients with allergy to apple and birch pollen were sensitized to a stable 9-kDa allergen in apple, identified as a lipid transfer protein that does not cross-react with Bet v 1 (19). These allergens do not seem to be equally important in northern Europe, where birch pollen is predominant (35). Several groups have shown that the clinically most important allergen in apples in northern Europe is Mal d 1, mainly due to the high degree of sequential homology between Bet v 1 and Mal d 1 (7–9, 14). Only one patient complained of occasional symptoms to apple juice without specifying whether the problem was fresh juice or commercial juice. This patient did not react to placebo. There was a significant relation between the onset of seasonal rhinitis and plant food allergy in this study (data not shown), indicating that the food allergy was related to birch pollen. Furthermore, the commercial apple juice in normal 1:5 dilution has been used for SPT in 21 birch- and apple-allergic patients (unpublished data) and did not lead to skin wheals of $\geq 7 \text{ mm}^2$.

Freshly grated apple, as used in model II, gave an acceptable sensitivity, but it was difficult to perform a precise dose titration; furthermore, grated fruit is very susceptible to oxidative degradation.

Lyophilization of foods is acceptable for DBPCFC and has been used for different allergen sources (36), but the process may change the allergenic profile of some foods (37). Use of freeze-dried material for challenges has some practical advantages over fresh apples. It allows a dose titration, the freeze-dried material can be stored, and the same batch of freeze-dried apple can be used for some time without the change of allergenicity seen during ripening and storage of apples (21, 22). Evaluated on six different drying sessions performed over 6 weeks, the described preparation of apples by direct freezing in liquid nitrogen before lyophilization seems to be practicable and reproducible. Since the process avoids addition of toxic compounds, the freeze-dried material can be used for both *in vivo* and *in vitro* tests. The freeze-dried material was able to elicit specific skin reactions and HR, as well as clinical symptoms, when used for oral challenges. Evaluated by HR and SPT, extracts of the freeze-dried apple were stable for at least 24 h.

Immunoblotting results indicated that Mal d 1 was preserved during the lyophilization and extraction processes.

In conclusion, the results of this study showed that it was possible to perform DBPCFC with apple. Three different models were compared, fresh apple juice, grated

apple, and freeze-dried apple, respectively. The first model had an unacceptably low sensitivity combined with a high rate of reactions to placebo. The sensitivity of the last two challenge models was comparable (0.74/0.60), and unspecific reactions to placebo were seen in 0/19 and 4/25 patients. It is highly desirable that the sensitivity of the DBPCFC with apple is increased further. Lyophilization of apples produces material useful for SPT, HR, and oral challenges, but an investigation of the long-time stability and the allergenic profile of the freeze-dried material is needed.

Acknowledgments

We thank the laboratory technician Rita Beder for her considerable contribution to the clinical work in this study and Dr Kay Fötisch of the Paul-Ehrlich-Institut for his help with the immunoblotting experiments. Dr Barbara Fahlbusch, University of Jena, Germany, is acknowledged for donation of the Mal d 1-specific monoclonal antibody. The patients were supplied with antihistamine tablets, kindly donated by Synthelabo Scandinavia A/S, Brøndby, Denmark.

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