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Standardization of food allergen extracts for skin prick test

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Abstract

The aim of the study was to standardize and evaluate technically optimized food allergen extracts for use in skin prick test (SPT). The standardization procedure comprised 36 allergic histories in 32 food allergic patients with 21 healthy, non-atopic individuals serving as controls. The patients had a history of allergic symptoms upon ingestion of either cow's milk ($n=3$), hen's egg ($n=9$), wheat ($n=4$), hazelnut ($n=14$) or cod ($n=6$). They also had specific IgE in serum to the food in question and a positive SPT with a fresh preparation of the food. The diagnosis had been confirmed by a double-blind, placebo-controlled food challenge, except for the hazelnut-allergic patients. The controls were subjected to an open food challenge with all the foods to ensure tolerance. The standardization was performed by means of titrated SPT in accordance with the guidelines on biological standardization from the Nordic Council on Medicine. Regression analysis of the skin wheal areas was performed for each patient and the median protein concentration of allergen preparation (median C_{h10}) eliciting a wheal area of the same size as histamine 10 mg/ml was calculated. The median C_{h10} was 0.56 mg/ml for milk, 0.88 mg/ml for egg, 5.4 mg/ml for wheat, 2.1 mg/ml for hazelnut and 0.017 mg/ml for the cod extract. The sensitivity of the median C_{h10} estimated from the SPT data was 1 for milk, 0.98 for egg, 1 for wheat, 1 for hazelnut and 0.87 for the cod extract. The allergenic activity of the hazelnut extract was further investigated by leukocyte histamine release (HR) and immunoblotting experiments using sera from 27 hazelnut allergic patients. The clinical sensitivity of the optimized hazelnut extract evaluated by HR was 0.78 compared to 0.30 for a commercially available hazelnut extract (Soluprick). Immunoblotting results showed a stronger IgE binding capacity and additional IgE-binding bands of the optimized hazelnut extract compared with the Soluprick extract.

Keywords: Food allergy; Skin prick test; Double-blind placebo-controlled food challenge; Leukocyte histamine release

1. Introduction

Skin prick test (SPT) is usually the first diagnostic procedure applied when food allergy is suspected, and consequently a high sensitivity of the test is mandatory. Most of the existing commercial food extracts are not standardized and the diagnostic efficiency is unknown.

The manufacturing of food allergen extracts for SPT comprises several problems. The selection of source material is important, especially for food of plant origin, which exists in different varieties. Further, the allergenic activity may change during ripening, storage and transportation [1]. Processing of the food may result in change of allergenic activity, because of instability of the allergens [2–4].

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Food often contains more than one allergen of clinical importance and only in a few cases have all allergens and their relative importance in a specific food been characterized. The allergenic activity, sensitivity and specificity of commercial available SPT extracts are known to differ between allergen sources and products [3,5–9]. In order to confirm the diagnosis of ''classical'' food allergy, that is food allergy not due to cross-reactions between pollen and food, it is necessary to compare the serological data with the clinical history of the patient and the final diagnosis must be based on the outcome of double-blind, placebo-controlled food challenges (DBPCFCs) [10,11]. Previous studies have shown that only about 30–60% of medical histories raising suspicion of classical food allergy, can be confirmed by DBPCFCs [12,13]. With regard to sensitization to pollen and concomitant cross-reactions with fruits, nuts, and vegetables, the clinical history seems to be of greater diagnostic value [14]. However, only a few studies have investigated this issue.

Most of the existing food allergen extracts are either not standardized or standardized serologically, that is by means of sera demonstrating specific immunoglobulin E (IgE) reacting to the allergen in question but without relevant clinical data on the patient.

The aim of this study was to standardize and evaluate new, technically optimized food allergen extracts for use in SPT.

2. Materials and methods

2.1. Design

The standardization was performed on patients with confirmed food allergy and in accordance with the guidelines on biological standardization from the Nordic Council on Medicine [15], using SPT for verification of in vivo activity and quantification of the potency of the food allergen extracts. Further the allergenic in vitro activity of the optimized hazelnut extract was evaluated by leucocyte histamine release and immunoblotting experiments.

The study was approved by the local ethics committee and all subjects gave written informed consent before entering the study.

2.2. Subjects

The standardization was performed on 32 food allergic patients (36 allergic histories) attending the Allergy Unit and 21 healthy, non-atopic controls. The patient group consisted of 25 women and seven men and the control group comprised 13 women and eight men.

The included patients had a history of allergic symptoms upon ingestion of either milk ($n=3$), egg ($n=9$), wheat ($n=4$), cod ($n=6$), or hazelnut ($n=14$), specific serum IgE to the food in question, and a positive SPT with a fresh preparation of the food.

For patients allergic to milk, egg, wheat, and cod the diagnosis had been confirmed by a DBPCFC. For the hazelnut allergic patients the diagnosis was based on a history involving allergic rhinoconjunctivitis in the birch pollen season and oral allergy syndrome (OAS) upon ingestion of hazelnut.

In case of confirmed allergy to more than one food, the results for each allergen were evaluated separately as one patient history.

The controls underwent an open food challenge with all the foods to ensure tolerance.

For the in vitro characterization of the hazelnut extract by HR and immunoblotting experiments, sera from patients with a clinical history of reactions to hazelnut and birch, positive SPT with fresh hazelnut, and positive specific IgE and/or positive SPT were used. Besides the 14 patients selected for the standardization, 12 patients with a convincing history of reaction to hazelnuts screened for the standardization, but either not fulfilling the inclusion criteria or the criteria for final analyses, were included. Further, one DBPCFC-positive patient was included.

2.3. Fresh food preparations for SPT

At the inclusion visit, SPT with a preparation of fresh food was performed. Cow's milk (3.5 g fat and 3.5 g protein/100 g) was used undiluted. Whole hen's egg white and yolk were mixed and then diluted with 0.9% NaCl to a 10% (v/v) solution. Wheat was used as a 10% (w/v) suspension in 0.9%

NaCl. SPT with fresh cod and fresh hazelnut were applied with the prick–prick technique [5,14].

2.4. Allergen extracts for SPT

The food allergen extracts were developed by ALK-Abelló (Hørsholm, Denmark).

The raw materials for each food allergen were carefully screened to select the material that had the best representation of allergens. The extraction and purification method was optimized in order to ensure a high yield of activity and a high allergen consistency from batch to batch. Each batch was standardized against a laboratory reference with regard to protein concentration, allergen composition and total allergenic activity.

The raw materials chosen for milk and egg were fresh low fat pasteurised milk and fresh eggs, both of organic quality. For hazelnut fresh not-roasted organic nuts with skin were used. For wheat and cod, the raw materials were wholemeal organic wheat flour and fresh codfish fillet, respectively.

The extraction and purification method was optimized for each food allergen individually. The method for hazelnut is given as a brief example: hazelnuts were ground, defatted and freeze–dried. The defatted hazelnuts were extracted in a ratio of 1:10 in a carbonate–saline buffer at pH 7.4. The extract was clarified by centrifugation and filtration and concentrated two times by ultrafiltration. Low-molecular-mass impurities were removed by diafiltration against five volumes of carbonate buffer, pH 7.4. The extract was subsequently filtered through a 0.22-μm filter, filled and freeze–dried.

The freeze–dried food allergen extracts were dissolved in carbonate buffer, pH 8.3 and diluted in suitable 10-fold dilutions and mixed with 50% glycerol.

2.5. Skin prick test procedure

The SPT was performed according to guidelines from the European Academy of Allergology and Clinical Immunology [16].

The SPT was applied at the volar surface of the lower forearm of the patient. Skin prick lancets with straight shoulders and a 1 mm tip were used (EWO Care, Sweden – ''Allergy-pricker'', Dome/Hollister-Stier [17]). Histamine dihydrocloride 10 mg/ml served as positive control and diluent (50% glycerol, saline and buffers) as negative control.

A stock solution of 10 mg/ml (total protein content) of each food allergen extract and five 10-fold dilutions were used.

The standardization was performed according to the principles in the Nordic guidelines on allergen standardization [15]: the concentrations of allergen extracts were selected in a preliminary test as the lowest concentration resulting in a positive skin reaction and the two higher concentrations. In the final test both allergen extracts and controls were applied in quadruplicate with a distance of more than 1.5 cm. The positive and negative controls were read 10 min after application, the allergen extracts after 15 min. The contour of the skin wheals were outlined with a pen and transferred by tape to a record sheet. The wheal areas were determined by computer scanning [18]. A skin prick test reaction was considered to be positive when the wheal diameter was $\geq$3 mm (area $\geq$7 mm^2).

Prior to SPT, medication was discontinued according to the guidelines [16]: short-acting antihistamines at least 2 days before, long-acting antihistamines 8 weeks and topical steroids 2–3 weeks before testing. None of the patients were treated with systemic steroid, hydroxyzine, ketotifen or tricyclic antidepressants a month prior to or during the study.

2.6. Specific IgE

Specific IgE was determined by the CAP-RAST system (Pharmacia & Upjohn, Uppsala, Sweden) according to specifications from the manufacturer.

2.7. Leukocyte histamine release

The histamine release experiment was performed as passive sensitization of basophil leukocytes in cord blood [19–21] using the same batch of cord blood for all sera.

For passive sensitization 3 ml of cord blood was washed once with Pipes buffer (Pipes-AMC (pH 7.4) [10 m*M* piperazine-*N*,*N*-(bis-ethane sulfonic acid), 140 m*M* sodium acetate, 5 m*M* potassium acetate,

0.6 m*M* $CaCl_2$, 1.1 m*M* $MgCl_2$, glucose 1 mg/ml, human serum albumin 0.3 mg/ml, heparin (Leo, Ballerup, Denmark) 15 IU/ml]) (Reference Laboratory, Copenhagen, Denmark) and centrifuged (400 *g* for 10 min). Plasma was removed and cells re-suspended in 1 ml of patient serum. The samples were incubated for 120 min at 37°C. The cells were then washed once with Pipes buffer, centrifuged (400 *g* for 10 min) and re-suspended in the buffer to a final volume of 3 ml. Il-3 (Genzyme, Cambridge, MA, USA) was added to the samples to a final concentration of 10 ng/ml blood before pre-incubation for 30 min at 37°C. Aliquots of 25 μl of allergen extract or anti-IgE were incubated with 25 μl of the passively sensitized cord blood in glass-fibre coated microtiter plates (Reference Laboratory) for 60 min at 37°C. All measurements were performed in duplicate. After washing with deionized water, incubation with sodium dodecyl sulfate (SDS) for 30 min at 37°C and finally washing with deionized water, the amount of histamine were then measured spectrofluorometrically by the *O*-phthaldialdehyde (OPT) method [20].

HRs were measured towards the optimized hazelnut extract (OHE), a commercially available Soluprick hazelnut extract (SPHE) from ALK-Abelló and a preparation of fresh hazelnut (FH) as described below.

Fresh hazelnut: an amount of 15 g fresh hazelnuts was crunched in a Stomacher 80 (Seward Lab System, UK) at high speed for 120 s with 30-ml Pipes buffer. After centrifugation at 2000 rpm for 10 min the supernatant was used as stock solution.

The optimized hazelnut extract: the extract was obtained as a lyophilized powder and was reconstituted in Pipes buffer to a protein concentration of 10 mg/ml.

The Soluprick hazelnut extract (1:100, w/v): the extract was suspended in 50% glycerol and for technical reasons it was not possible to run in a HR-test without further dilution. Therefore all three hazelnut extracts were diluted with Pipes buffer a further 10 times before use in the HR experiment.

Anti IgE (Behringwerke, Marbourg, Germany) was used as positive control in the following final concentrations: 400, 40 and 4 U/ml. The allergen extracts were added to the plates in nine 3.5-fold dilutions. A histamine release $\geq$13 ng/ml was considered positive.

In order to exclude unspecific release, identical experiments were performed with serum from a healthy, non-atopic individual: all nine dilutions of the allergen extracts consistently produced a release $\leq$10 ng/ml.

2.8. Immunoblotting

The allergenic profile of the hazelnut extracts (SPHE, OHE, FH) and the IgE-reactivity of 27 hazelnut allergic patients were characterized by means of SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting.

A serum without specific IgE against hazelnut was tested as negative control.

The protein contents of the extracts were measured using the method of Lowry et al. [22] (SPHE) and the BCA method (BCA Protein Assay, Bichinchoninic acid kit for Protein Determination) [23] (OHE and FH). The protein determination of the stock solutions showed 1.3 mg/ml (SPHE), 8.8 mg/ml (OHE) and 9.7 mg/ml (FH).

SDS–PAGE gels were run on Bio-Rad Protean II equipment (16×12 cm gel) using 16% acrylamide in the separation gel and 7% in the stacking gel [24].

The extracts were prepared in sample buffer (150 m*M* Tris–HCl, pH 7.25, 324 m*M* dithiothreitol, 1% SDS, bromphenol blue), heated to 100°C for 10 min and run in broad slits (11.5 cm) with an M_r marker included on each side of all slits. The proteins were transferred to nitro-cellulose blotting membranes (0.2 μm; Sartorius, Göttingen, Germany) using a semi-dry blotting cell [25].

After electroblotting, the nitrocellulose membranes were cut into strips of 0.5 cm. From the protein determinations of the extracts the amounts of protein on the strips (0.5 cm) were calculated as 11.7 μg (SPHE), 15.8 μg (OHE) and 17.5 μg (FH).

Each strip was incubated with 150 μl serum diluted to 4 ml in incubation buffer [phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), 0.005% Tween 20]. Incubation took place overnight at room temperature and after washing in buffer (5 m*M* Tris, 150 m*M* NaCl, 0.05% Tween 20, pH 10.3), IgE-binding was detected with

I^{125}-aIgE (Pharmacia & Upjohn, Uppsala, Sweden) 70 000 cpm/strip in 4 ml incubation buffer overnight at room temperature. The strips were washed and dried, mounted in X-ray cassettes and exposed for 30 days at −80°C.

Furthermore, the three extracts were examined by crossed immuno electrophoresis using a rabbit antibody against OHE (data not shown).

2.9. Statistics

For determination of C_{h10} for each extract, the median concentration of allergen eliciting a skin wheal of the same size as histamine 10 mg/ml was calculated.

Regression analysis including the log value of all four skin wheal areas of each concentration was performed for each patient. Only results from individual patients fulfilling the following criteria were accepted for the final analysis of the median C_{h10}: (1) geometric mean of the wheal reactions provoked by either of two highest concentrations of allergen extract ≥ 7 mm^2. (2) Geometric mean of the wheal reactions provoked by histamine dihydrocloride 10 mg/ml ≥ 7 mm^2. (3) Geometric mean of the wheal reactions provoked by the negative control <7 mm^2. (4) Slope of regression≥ 0.1. (5) The pooled standard deviation of the four log wheal areas obtained with all concentrations of the allergen extract in all patients <0.4 [16].

Median C_{h10} of the different allergen extracts were compared by Sigmastat 1.0 with non-parametric methods, Kruskal–Wallis one-way analysis of variance on Ranks, Dunn's Method.

Sensitivity/specificity evaluated by SPT and HR were calculated according to Ref. [26].

Diseased was defined as a positive clinical history of food allergy combined with a positive SPT with a fresh preparation of the food.

The sensitivity of the median C_{h10} was obtained by interpolation from the results for the concentrations lower and higher than the median $C_{h10.}$

3. Results

3.1. Standardization

The characteristics of the patients and the controls are shown in Table 1. The results of the SPT with fresh food and specific IgE are given as median and range. Only the patients fulfilling inclusion criteria for standardization and valid for final analysis have been included in the table. In addition 23 allergic histories were evaluated: 12 did not fulfill the inclusion criteria for biological standardization (no specific IgE in serum), for 10 patients the SPT data did not fulfill the criteria for final analysis, and one patient dropped out of the study for personal reasons before the final SPT. The clinical history of the patients included the following manifestations upon ingestion of the food in question: OAS (78% of the patients), gastrointestinal symptoms such as nausea, stomach pain, vomiting, diarrhoea (39%), rhinocon-

Table 1
Characteristics of patients and controls used for standardization

Allergen	No. of patients	Sex (F/M)	Age (years)	SPT-fresh (mm^2)	Specific IgE (kU/l)
Milk	3	2/1	28 (23–33)	48 (31–74)	176 (8.5–220)
Egg	9	6/3	40 (19–71)	39 (12–179)	3.2 (1.2–14)
Wheat	4	4/0	42 (42–53)	23 (11–32)	2.1 (1–22)
Hazelnut	14	11/3	31 (20–51)	46 (11–101)	1.5 (0–19)
Cod	6	5/1	30 (24–36)	102 (77–226)	8.5 (2.6–6.69)
Controls	21	13/8	36 (20–55)	0 (0–0)	Not done

Results for age are given as mean (range), SPT and specific IgE as median (range). Specific IgE was measured by the CAP system (Pharmacia & Upjohn, Uppsala, Sweden). Specific IgE for egg is represented as specific IgE against egg white except for one patient, who was negative to egg white but positive to egg yolk. One hazelnut allergic patient had no specific IgE to hazelnut measured by the CAP system but had positive IgE measured by Magic Lite (ALK-Abello, Horsholm, Denmark).

junctivitis (36%), asthma (36%), urticaria (26%), angio-edema (17%), worsening of atopic dermatitis (14%), and severe systemic reaction (6%).

3.2. Median C_{h10}

The C_{h10} for each patient is shown in Fig. 1. The median (range) of the allergen preparations was: 0.56 (0.2–1.9) mg/ml for milk, 0.88 (0.017–11) mg/ml for egg, 5.4 (1.1–13) mg/ml for wheat, 2.1 (0.43–9) mg/ml for hazelnut, and 0.017 (0.0003–0.11) mg/ml for the cod extract. Calculation of a 95% confidence interval was only possible for the hazelnut extract: 1.2–7.2 mg/ml.

The figure shows variation in C_{h10} between food allergen sources and between patients with allergy to the same food indicated by the median and range. The following differences were statistically significant ($P<0.05$): C_{h10} wheat$>C_{h10}$ cod; C_{h10} hazelnut$>$cod. There were no significant differences between wheat and milk, or egg and cod. The applied statistical software did not allow calculation on the remaining combinations. The pooled standard deviation of the four log wheal areas, obtained with all concentrations of the extracts was below 0.4 for milk, egg, hazelnut, and cod. The pooled standard deviation for the wheat extract was 0.47.

3.3. Clinical sensitivity and specificity

The sensitivity of each allergen extract concentration and the median C_{h10} was calculated. Median C_{h10}, sensitivity and specificity results for each allergen are shown in Fig. 2.

The sensitivity of the median C_{h10}, calculated from interpolation of the curves of the allergen extract concentration above and below the median C_{h10}, was 1 for the milk extract, 0.98 for the egg

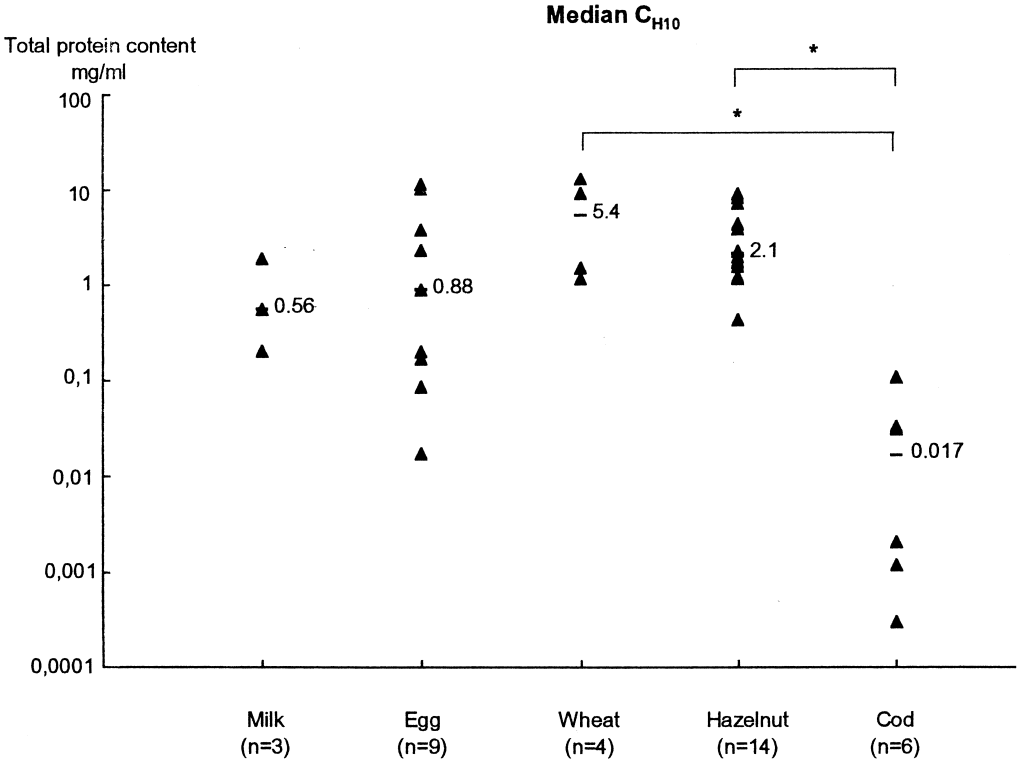

Fig. 1. Median C_{h10} of the allergen extracts (–) and C_{h10} for each patient (▲). The median (range) (mg/ml) of the allergen preparations: 0.56 (0.2–1.9) mg/ml for milk, 0.88 (0.017–11) mg/ml for egg, 5.4 (1.1–13) mg/ml for wheat, 2.1 (0.43–9) mg/ml for hazelnut and 0.017 (0.0003–0.11) mg/ml for the cod extract. *$P<0.05$.

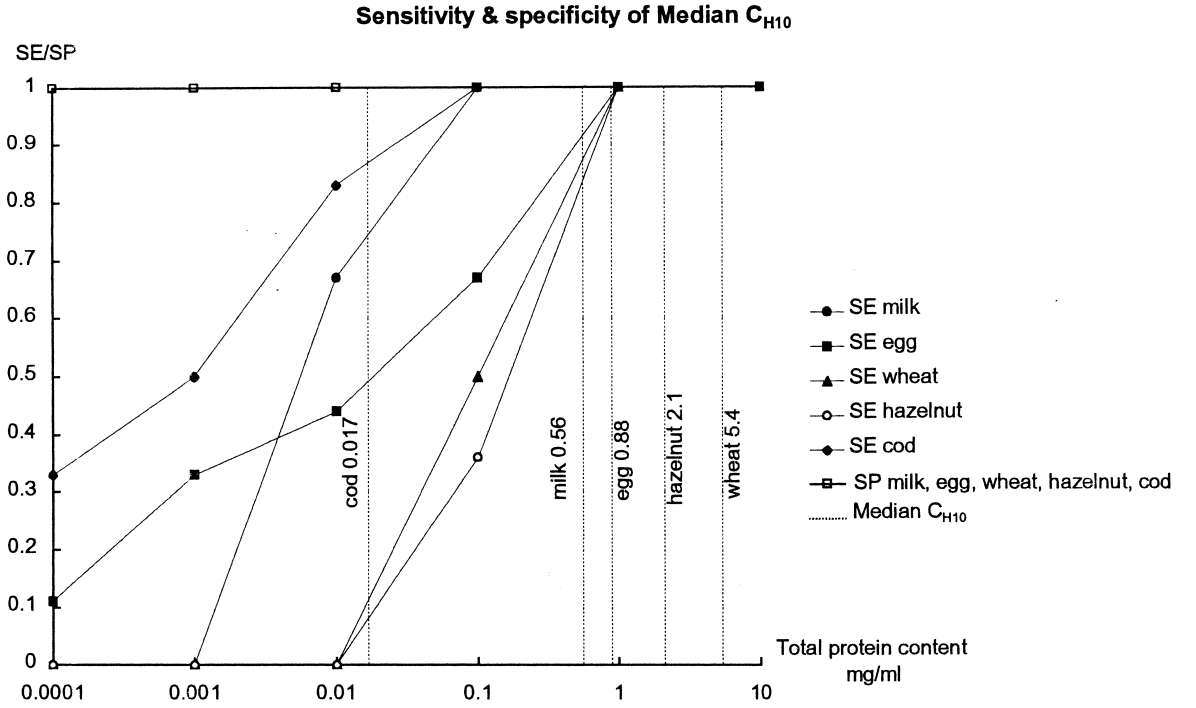

Fig. 2. Median C_{h10} sensitivity (SE) and specificity (SP) results for all allergen extracts. The sensitivity is presented as the frequency of patients eliciting wheal areas ≥ 7 mm^2 to the applied concentration of allergen extract. The sensitivity of the median C_{h10} (dotted line) was obtained by interpolation from the sensitivity of the concentrations lower and higher than the median C_{h10}.

extract, 1 for the wheat extract, 1 for the hazelnut extract and finally 0.87 for the cod extract. The sensitivity of the actually applied extract concentration just below the median C_{h10} was 1 for the milk extract, 0.67 for the egg extract, 1 for the wheat extract, 1 for the hazelnut extract and 0.83 for the cod extract. None of the controls had positive SPT results for either the fresh foods or the extracts, applied in the highest protein concentration (10 mg/ml), thus giving a specificity of 1 for all allergens in this material.

3.4. Safety

No adverse reactions were registered after the SPT with the allergen extracts in either the patients or the controls. A few patients developed a local, late-phase skin reaction after the SPT with fresh food. Six patients, five of which were included in the standardization, reported in all seven adverse reactions: one reaction to egg, two to wheat and four to hazelnut. A single patient reported generalized pruritus of the skin several hours after the SPT with fresh wheat and fresh hazelnut. None of the reactions were regarded as serious. Two patients treated the symptoms with antihistamine tablets, and the patient with pruritus normally used prednisolone tablets to cure allergic symptoms and took a single dose of 2.5 mg of prednisolone. The control group did not report any adverse events during the SPT with fresh food.

3.5. In vitro activity of the hazelnut extract

HR against FH, the SPHE, and the OHE are shown in Figs. 3 and 4. For technical reasons it was not possible to test more than 23 of the 27 hazelnut sera in one set-up of the HR test. The results in Fig. 3 are presented as the protein content of the 3.5-fold dilution of the allergen extract or fresh preparation at which the release was 50% of the maximal release (1/2 max HR), e.g., the lower the protein content at 1/2 max HR, the higher allergenic potency of the

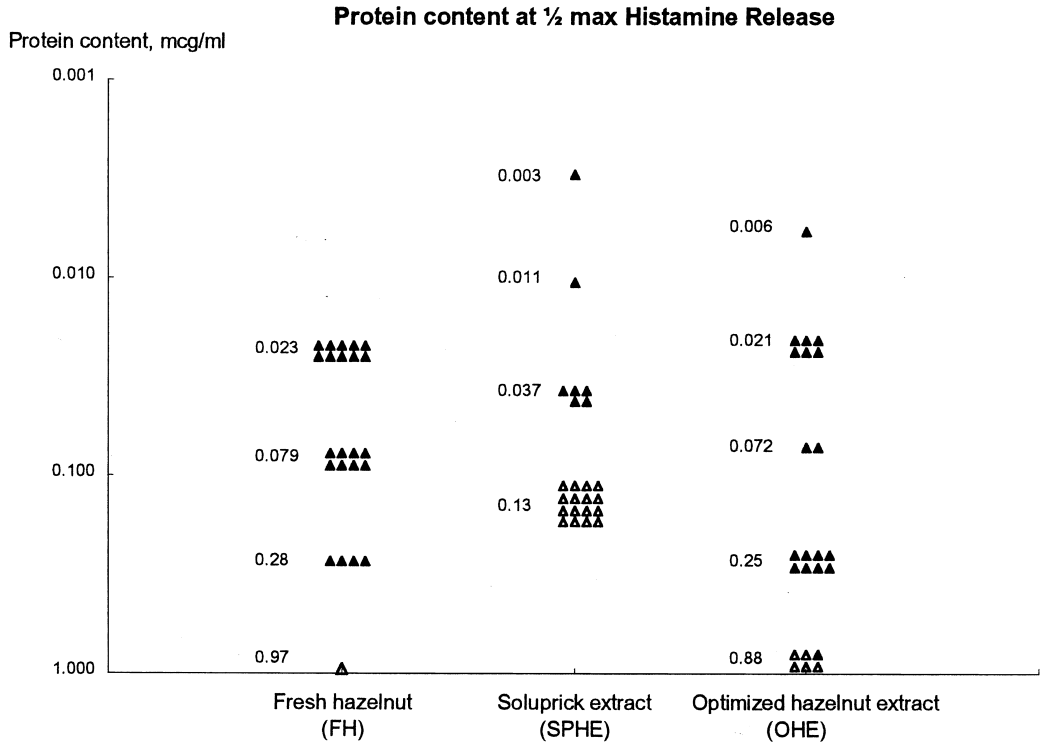

Fig. 3. Histamine release against fresh hazelnut (FH), the Soluprick hazelnut extract (SPHE) and the new optimized hazelnut extract (OHE). The figure illustrates for each patient (▲) the protein content of the 3.5-fold dilution of the hazelnut extracts, at which the HR was 50% of the maximal release (1/2 max HR). The patients with a release <13 ng/ml (△) are plotted at the protein content of the stock solutions of each extract: 0.97 (FH), 0.13 (SPHE) and 0.88 (OHE).

extract. Patient Nos. 1, 16, and 5 of the 23 patients had no significant release (<13 ng/ml) to FH, SPHE, and OHE, respectively. The HR negative patients are plotted as open symbols at the protein content of the stock solutions used for HR: 0.97 mg/ml (FH), 0.13 mg/ml (SPHE), and 0.88 mg/ml (OHE), respectively.

Fig. 4 illustrates the relation between 1/2 max HR to FH, SPHE, and OHE for each of the 23 patients tested. The delta 1/2 max HR is the difference in titer, i.e., patient No. 1 responded to one titer step lower in OHE, but three titer steps lower in SPHE. The figure shows that there no constant relation in 1/2 max release between the extracts from patient to patient.

Further, the clinical SE evaluated by the HR results was calculated. The true diagnosis was defined as a clinical history of allergy to hazelnuts and birch combined with a positive SPT with fresh hazelnut. The sensitivity of OHE was 0.78 (18/23), compared with 0.30 (7/23) for SPHE and 0.96 (22/23) for FH. The different protein content of the hazelnut extracts however, influences both the release data and the calculation of the sensitivity.

Immunoblotting results are shown in Fig. 5.

IgE-blotting showed a major IgE-binding band at approximately M_r 19 000 to which 23 of the 27 sera reacted. IgE-binding bands were also seen in the regions of M_r 12 000–14 000, 29 000–33 000 and 38 000–40 000. One patient reacted to bands at M_r 9000 and 57 000. Two bands at M_r 24 000 and 43 000 exhibited binding of all sera including the negative control.

IgE-binding patterns of OHE and FH were almost identical, whereas SPHE exhibited less IgE-binding protein bands. These results were confirmed by crossed immuno electrophoresis, that showed great similarities between OHE and FH, in concentration

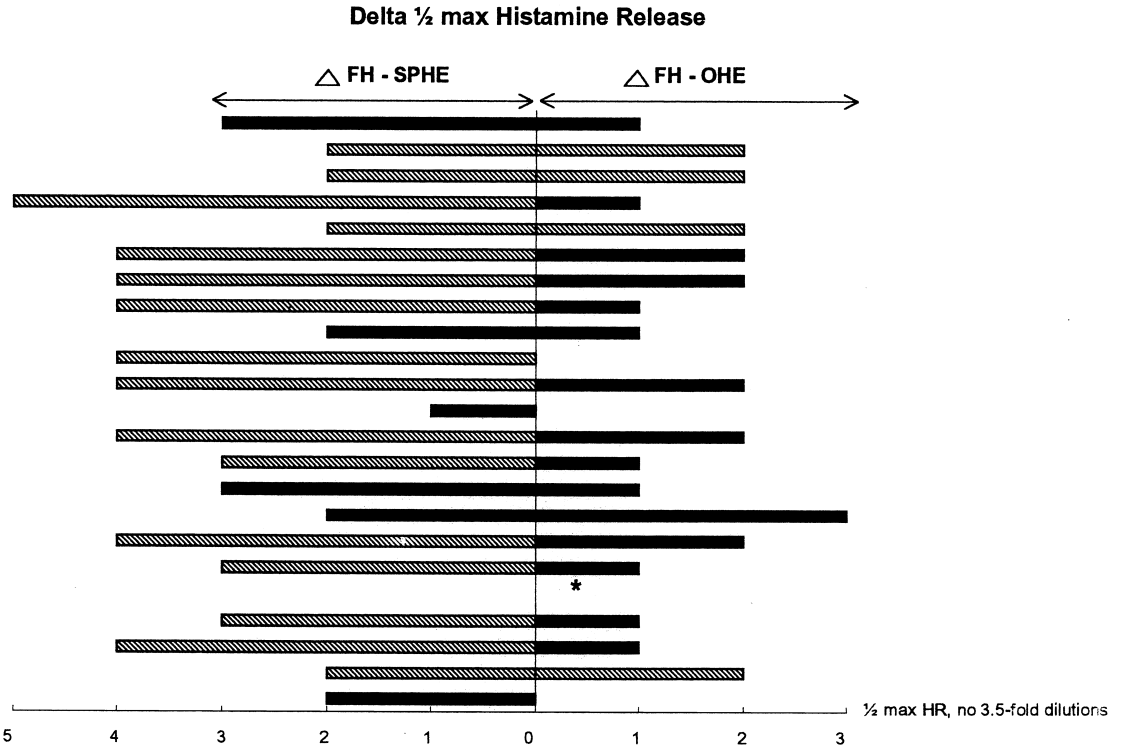

Fig. 4. Histamine release against fresh hazelnut (FH), the Soluprick hazelnut extract (SPHE) and the optimized hazelnut extract (OHE). The figure illustrates the relation in 1/2 max HR between FH, SPHE and OHE for each of the 23 patients tested. The delta 1/2 max HR is the difference in number of 3.5-fold dilutions corresponding to 50% of the maximal release between FH and SPHE of OHE. The difference between FH and SPHE is shown at the left side of the *y*-axis and the difference between FH and OHE at the right side. The black bars indicate significant release $\geq$13 ng/ml, the hatched bars indicate release $<$13 ng/ml. ★ is a patient with release $<$13 ng/ml to all three extracts.

as well as in composition, as compared to the much weaker SPHE, that seems to lack several components.

4. Discussion

Due to the lack of standardized food allergen extracts, use of fresh food for the SPT has been recommended in the diagnosis of food allergy [16]. Several studies have shown that both the sensitivity and the specificity of the SPT are enhanced when fresh food are compared with the available commercial extracts for use in the SPT [5,8,12,27,28].

The use of non-processed, fresh food poses some problems: it is difficult and for some allergen sources, impossible to standardize the procedure: Most fruit and vegetables exist in different varieties [29] and also exhibit seasonal variation [1]. Further it is necessary to establish the optimal dilution of liquid allergen sources, e.g., milk in order to compare results.

In this study no serious, adverse events were seen after the SPT either with the allergen extracts or fresh food, but some patients experienced local discomfort at the application site after the SPT with fresh food. There are a few reports of severe, systemic reactions after the SPT [9] and in some highly sensitized patients it may therefore be important to know the exact concentration of protein in a food preparation and be able to titrate the dose as a safety precaution. For some foods, especially solid foods, it is not possible to make a titration without processing and thereby potentially alter the allergenic

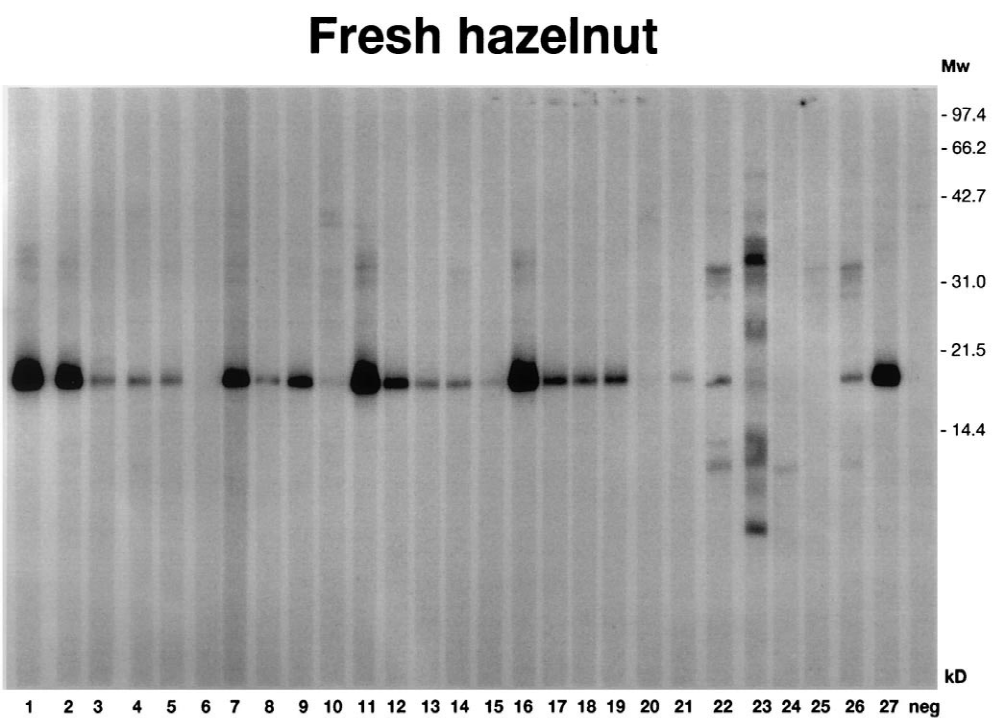

Fig. 5. The allergenic profile of the hazelnut extracts (SPHE, OHE, FH) and the IgE-reactivity of 27 hazelnut allergic patients and a negative control. IgE-blotting (SDS–PAGE) showed a major IgE-binding band at approximately M_r 19 000 to which 23 of the 27 sera reacted. Serum from patient Nos. 6, 20, 24 and 25 did not bind to this band. IgE-binding bands were also seen in the regions of M_r 12 000–14 000, 29 000–33 000 and 38 000–40 000. One patient (No. 23) reacted to bands at M_r 9000 and 57 000. Two bands at M_r 24 000 and 43 000 exhibited binding of all sera including the negative control. The protein amounts on the strips (0.5 cm) were SPHE: 11.7 μg, OHE: 15.8 μg and FH: 17.5 μg.

activity. Food allergen extracts may therefore represent a more dynamic and safe tool than fresh food and standardization of food allergen extracts will improve the overall diagnostic abilities concerning food allergy, increasing the diagnostic value of both in vivo and in vitro tests.

The Nordic Committee on Allergen Standardization and others proposes standardization of food allergen extracts by determination of the major allergen content instead of biological standardization using skin tests, because of the limited number of patients and the inhomogeneity in age, sensitivity and symptoms [16,30,31]. The American Academy of Allergy, Asthma and Immunology on the other hand, suggests that at least extracts of some of the most common food allergen sources, including cow's milk, hen's egg, shrimp, and peanut should be susceptible to biological standardization [32].

The immunoblotting of the hazelnut extracts revealed that the majority of the 27 patients, but not all 14 included in the standardization, reacted to a protein band with a molecular mass of approximately 19 000. Thus two of the 14 patients, eligible for the standardization procedure, did not react to the M_r 19 000-band. This protein band is compatible with a band in hazelnuts found by several groups [33,34] with IgE-binding properties similar to Cor a 1, the predominant allergen in hazel pollen. If it is decided to standardize food allergen extracts by determination of major allergen content, it is important to ensure the presence of quantitatively less important allergens in the extracts. Moreover, it will be important to evaluate the sensitivity and specificity of such ''major allergen extracts''.

The HR showed no quantitative differences that could not be explained by difference in protein

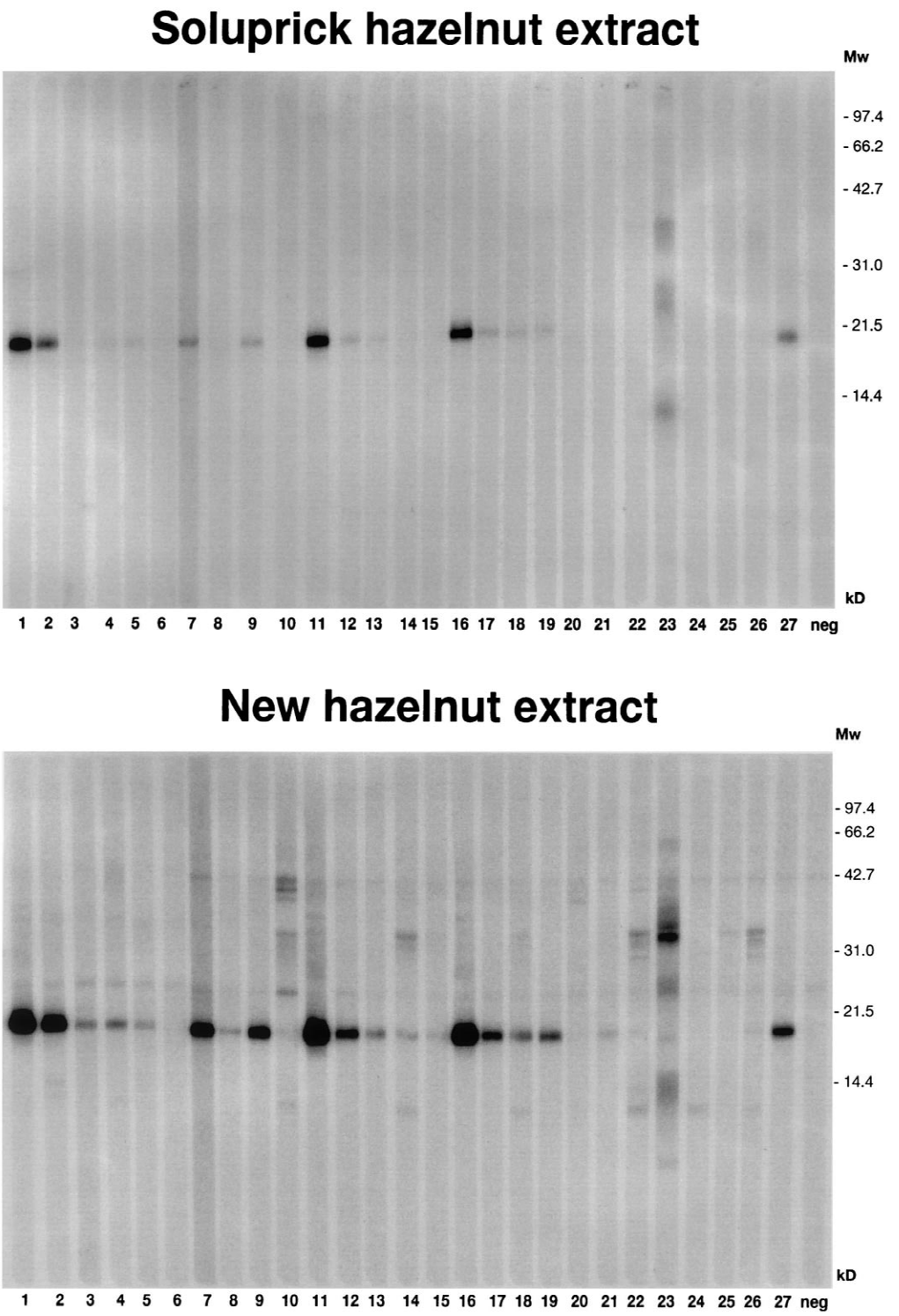

Fig. 5. (*continued*)

content between the extracts – a fact that could imply at least partial identity in allergenic profile between the fresh preparation of hazelnut and the two allergen extracts. On the other hand, the HR results could not confirm a proportional relation between the hazelnut extracts from patient to patient. Further the immunoblotting experiments showed that the IgE-binding pattern of the optimized hazelnut extract and the fresh preparation of hazelnut were almost identical, whereas the Soluprick hazelnut extract gave a weaker binding and exhibited less IgE-binding protein bands. These results might indicate possible qualitative differences between the extracts.

Evaluated by HR test, the allergenic activity and thereby the sensitivity of the new hazelnut extract was improved considerably in comparison with the Soluprick extract – an increase in sensitivity from 0.3 to 0.78, but not matching that of the fresh hazelnut extract (0.96).

According to the guidelines [15] only medium sensitized patients should be included for biological standardization, but because the SPT is used as the initial screening of patients with a positive case history, a high sensitivity is mandatory, even if it means a somewhat lower specificity. In this study, we chose to include patients despite low amounts of specific IgE and small skin reaction to a preparation of fresh food, to ensure that no food allergic patient was overlooked. The sensitivity of the median C_{h10} calculated from the results of the SPT with the different allergen extract concentrations applied was below 1 for the egg and cod extracts, 0.98 and 0.87, respectively. This means of cause that not all egg and cod allergic patients fulfilling the criteria for standardization would have been identified as positive by testing with the extracts.

The results of the present study show a rather large variation in the allergen concentration corresponding to histamine 10 mg/ml, both between allergen systems and between the patients within the allergen groups. This fact supports the notion that each food allergen source is unique and that is it impossible to infer the diagnostic efficiency from one allergen extract to another. The variation also imply that it could be a problem to use the same allergen concentration for all patients suspected of food allergy, if the least sensitized patients require a concentration so high that the most highly sensitized patients would experience adverse reactions. A solution to this problem could be manufacturing of extracts, standardized according to guidelines but corresponding to different concentrations of histamine, e.g., three preparations corresponding to histamine 1, 10 and 100 mg/ml, or alternatively if the extracts were available in different dilutions, 1:10 and 1:100. These procedures may increase sensitivity without compromising the safety of the SPT procedure.

To our knowledge no studies on biological standardization of food allergen extracts have been published until now, and this study clearly documents the difficulties in recruiting enough patients, to meet the requirements for biological standardization as outlined by the guidelines. It should be noted that the requirements for a true diagnostic trial were not fulfilled since the patients in this study were highly selected and limited in number, due to the relative low prevalence of classical food allergy in the adult population and the requirements for biological standardization. Further the control group consisted of non-atopic individuals only. To reach an optimal number of patients for all the allergens tested and to evaluate the true diagnostic sensitivity of the extracts a multi-center study may be needed.

In conclusion the sensitivity of the calculated median C_{h10} for the SPT was high, but not optimal for all the allergens tested. No unspecific skin reactions were seen when the extracts were applied in the highest concentration in healthy controls and the use of a concentration above the median C_{h10} may therefore be favorable and would not affect the specificity negatively.

5. Nomenclature

DBPCFC	Double-blind, placebo-controlled food challenge
FH	Fresh hazelnut
1/2 max HR	Allergen extract concentration providing a histamine release at 50% of maximal release
HR	Leukocyte histamine release
Median C_{h10}	The median concentration of allergen extract eliciting a skin wheal of the

	same size as histamine 10 mg/ml, median C_{h10}~10 000 BU (biological units)~10 HEP (histamine equivalent prick)
OAS	Oral allergy syndrome
OHE	Optimized hazelnut extract
SPHE	Soluprick hazelnut extract
SE	Sensitivity
SP	Specificity
SPT	Skin prick test

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