

Codfish allergy in adults. Specific tests for IgE and histamine release vs double-blind, placebo-controlled challenges

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Summary

Background At present, several *in vitro* tests for immunoglobulin E (IgE)-mediated food allergy are available. An estimation of the diagnostic accuracy of the various tests used in predicting clinical sensitivity to codfish in a well-characterized allergic material is necessary.

Objectives To compare the diagnostic value of four specific IgE tests, and histamine release from basophils (HR) in identifying clinical type I allergy to codfish. As a true diagnosis, double-blind, placebo-controlled food challenges (DBPCFC) were employed.

Methods Eight clinically codfish-allergic adult patients were investigated together with 30 codfish-tolerant control subjects for evidence of codfish-specific reactivity by Phadebas RAST® (PHA), Pharmacia CAP System RAST® (CAP), Magic® Lite (ML) and HR. To characterize the diagnostic properties of a freshly prepared raw codfish extract, experiments were conducted employing an in-house radioallergosorbent test (RAST), the Maxisorp RAST (MAXI) and HR. Finally, protein profile and IgE-reacting allergens were detected by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Results The sensitivities of HR with commercial extract and the three commercially available specific IgE analyses were 0.83 and 1.00 respectively. Specificities were 1.00 (HR) and 0.87–1.00 (specific IgE tests). Freshly prepared codfish extracts improved the sensitivity of HR. SDS-PAGE revealed ≈29 bands (<14.3–200 kDa) including a band of 12–13 kDa, and in immunoblotting 18 sera identified 17 IgE-binding bands. The protein migrating at 12–13 kDa was identified in the fresh codfish extract tested with sera from all clinical codfish allergics, while no significant reaction was seen in the control subjects.

Conclusion Based on the small number of adult patients included in our study, the *in vitro* assays with commercial and fresh extracts have high sensitivity and are acceptable for screening for codfish allergy. Specificity of Phadebas, CAP, and our in-house RAST was less than unity, whereas ML and strong binding of IgE to a 12–13 kDa protein completely matches DBPCFC results, and thus seems sufficient for establishing the diagnosis.

Keywords: adult, double-blind method, fishes, food hypersensitivity, histamine liberation, IgE, immunoblotting, radioallergosorbent test, sodium dodecyl sulfate

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Introduction

From a historical perspective, hypersensitivity to fish plays an important role in studies of allergy and allergic diseases. In 1921 Prausnitz and Küstner [1] called attention to fish

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allergy by demonstrating that skin-test reactivity could be transferred to a non-allergic subject with serum from a fish-allergic patient. The classic passive transfer test, or Prausnitz-Küstner test, has been largely replaced by *in vitro* assays for fish allergy.

Double-blind, placebo-controlled food challenge (DBPCFC) is considered to be the only reliable procedure for diagnosing food allergies [2,3], and so far it has not been possible to substitute DBPCFC by any other test, neither alone nor in combination. By comparison to the outcome of DBPCFC, the ability of a given test (skin prick test, IgE-assays, histamine release test) to predict symptomatic hypersensitivity (diagnostic sensitivity (SE) and specificity (SP)) can be determined [2]. Varying sensitivities of the tests have been reported [4–6] mainly due to differences in allergen quality, in patient selection, or in the nature of the food in question.

Most studies of fish allergy have been performed on paediatric patients, where comparisons of *in vivo* and *in vitro* tests generally have shown the latter to be less sensitive [5,7–9], but only in few studies have DBPCFCs been performed. However, the quality of the extract used plays an important role in the diagnostic accuracy of the tests. It has been shown in a study of fish allergic children and young adults that a positive skin prick test (SPT) or *in vitro* test did not necessarily correlate with symptomatic fish allergy [10]; although having positive tests, they were clinically negative to the fish species tested. When using the same extract in radioallergen sorbent test (RAST) and for ingestion [11], a 100% correlation was found between a positive RAST and the presence of clinical allergy to codfish in children. A highly purified codfish allergen was used.

Therefore, the aim of this study was to evaluate the SE and SP of *in vitro* tests in clinically codfish-allergic adult patients (i.e. confirmed by DBPCFC with codfish). To ensure optimal quality of the allergen material, SDS-PAGE and immunoblotting were applied on the allergen extract, prepared from fresh codfish and used in an in-house RAST, and by histamine release test.

Materials and methods

Patients and control subjects

Eight clinically codfish allergic adult patients (ranging in age from 21 to 31 years) were selected for this study. Seven of these patients have been described earlier [12], while one patient was newly included. All patients reported objectively measurable symptoms within 30 min after ingestion of small amounts of codfish. Food hypersensitivity was diagnosed in all eight by titrated double-blind, placebo-controlled oral codfish challenges [12], whereby all eight reacted showing

allergic symptoms during the active challenges, and without any symptoms during the corresponding placebo challenges. Furthermore, they all had a positive SPT with the same batch of commercially available, glycerinated codfish extract, 1:20 w/v (Soluprick[®], ALK Hørsholm, Denmark), performed according to the EAACI Guidelines [13].

Thirty codfish-tolerant adult subjects served as a control group. The control group was comprised of four subgroups, of which three had never experienced allergic symptoms upon ingestion of codfish: eight non-atopics; seven pollen allergics (proved by case histories and SPT or specific IgE tests); and 12 with atopic dermatitis [14], six having serum-IgE concentrations >2000 kIU/l and six <2000 kIU/l. The fourth subgroup consisted of three adults who were suspected of having immediate allergic reactions upon ingestion of codfish, but who were all found to be codfish-tolerant (DBPCFC-negative), according to DBPCFC performed as mentioned above [12]. One of these three challenge-negatives reacted positively in SPT with commercial extract (Table 1), whilst the remaining 27 control subjects had negative skin prick tests. Since these 27 subjects ingested codfish regularly without any adverse reactions, they did not undergo DBPCFC, but were classified as challenge-negatives.

Blood was drawn from all 38 subjects at the initial visit: the heparinized whole blood was used within 24 h for histamine release test, and the sera were stored at –20°C for codfish-specific IgE analyses and immunoblotting.

Challenges

Fresh, raw codfish was used for titrated, oral DBPCFC. All challenges were performed double-blind and placebo-controlled with increasing doses between 5 µg and 5 g of fresh codfish (cumulated 6.7 g) masked in a placebo vehicle (concentrated blackcurrant juice without dyes or preservatives in distilled water, 1:4) or pure placebo vehicle in identical numbers and volumes. The challenge procedure has previously been described in detail [12]. A positive reaction was defined as objective signs to an active challenge without having any symptoms after the corresponding placebo challenge. In case of subjective reactions to the active challenge, three crossover trials with reactions developing only during the active challenge were necessary. Challenges with a negative outcome were not followed by an open challenge.

Subjective symptoms, consisting of oral itching (all patients), nausea (two patients) or abdominal pain (one patient) were elicited at 6 mg–6.7 g. The following objective signs were observed (based on a clinical evaluation); erythema (four patients), blisters (four patients), angioedema (three patients), oropharyngeal swelling (two patients), asthma (≥ 20% drop in FEV₁, one patient), and vomiting (one patient) occurred between 56 mg and 6.7 g.

Table 1. Demographical and immunological characteristics of the study group. A negative case history indicates regular ingestion of codfish without any adverse reactions. Skin prick tests (SPT) were performed in quadruplicate on the subjects' forearms. Positive control: histamine dihydrochloride 10 mg/ml. Negative control: diluent of glycerol/sodium chloride. Positive reaction: a wheal net diameter 3 mm larger than that of the negative control

	Case history +/-	Age	Sex	Total IgE (kIU/l)		Positive SPT
				Mean	Range	
8 clinically codfish-allergic patients (DBPCFC-positives)	+	21-31	6F, 2M	2315	29-14600	8/8
3 DBPCFC-negatives	+	23-28	1F, 2M	5362	83-15900	1/3
8 non-atopics	-	26-43	7F, 1M	20	3-72	0/8
7 pollen allergics	-	25-48	2F, 5M	106	12-401	0/7
12 atopic dermatitis:						
6 < 2000 kIU/l	-	17-32	5F, 1M	808	5-1883	0/6
6 > 2000 kIU/l	-	20-38	3F, 3M	8208	2153-15834	0/6

Anaphylaxis occurred in one patient that was challenged outside this protocol [12].

Allergen extracts

Commercial, glycerinated codfish extract (726, Soluprick[®], ALK Hørsholm, Denmark) 1:20 w/v was used for histamine release test.

Fresh, raw white meat of codfish (stored at -20 °C for a maximum of 7 days before use) homogenized in PBS (without azide, 10 mmol, pH 7.4) or distilled water (50 mg wet weight/ml) for 2 × 3 min using a thermostatically controlled Waring blender (Waring Products Div., New Hartford, Connecticut, USA) was used. For Maxisorp the extract was centrifuged (1800 g, 15 min), while the suspension was used for histamine release test, SDS-PAGE and immunoblotting.

Total IgE

Total serum-IgE was measured in all patients and control subjects by the PRIST kit (Pharmacia, Uppsala, Sweden), and concentrations were expressed as kIU/l.

Commercially available tests for specific IgE

Codfish-specific IgE analyses (f3) were measured in duplicate by Phadebas RAST[®] (PHA) (Pharmacia Diagnostics, Uppsala, Sweden) and results were expressed in PRU/ml (measuring range 0.35-17.5 PRU/ml).

The Pharmacia CAP System RAST[®] (CAP) (f3), was performed in duplicate according to the instructions of the manufacturer. Results were expressed in kU/l (measuring range 0.35-100 kU/l).

Magic[®] Lite (ML) was performed in duplicate (726) using the chemiluminescent assay from ALK (Hørsholm, Denmark). Results were given in SU/ml (measuring range 1.43-300 SU/ml).

Maxisorp RAST

The Maxisorp RAST (MAXI) was performed according to the method previously described [15]. The Maxisorp[®] polystyrene test tubes (Nunc, Roskilde, Denmark) were coated by addition of 300 µl of allergen diluted in PBS with azide (pH 7.4), and a few were coated with buffer. These were incubated overnight at 37 °C.

The serum samples to be tested were diluted 1:10 in phosphate buffered saline (PBS) 1/15 mol/l, pH 7.0, containing azide 0.01% and Tween 20 0.1%) and 300 µl were added to the allergen-coated tubes and incubated overnight at room temperature on a vibrator. After incubation, 300 µl of ¹²⁵I anti-IgE (Pharmacia, Uppsala, Sweden), diluted 1:12 (≈10000 cpm) was added to the tubes, incubated overnight at room temperature on a vibrator, and finally were counted for 5 min in a gamma-scintillation counter. Between each step the test-tubes were washed three times in PBS.

A pool of RAST-negative sera from blood donors served as a negative reference serum (sera were negative to pools of pollen, fungi and animal dandruffs).

Results were obtained from duplicate analyses and means were expressed as percentage-binding = sample/total cpm.

In dose-response experiments the optimal allergen concentration of fresh codfish in coating the surface of the test tubes was initially determined to be 100 µg/ml (results not shown).

Histamine release test — commercial and fresh extracts

Histamine release from basophil leucocytes (HR) was performed as previously described [16,17]. Heparinized blood (20 ml) was drawn from each patient and control subject, stored at room temperature, and used within 24 h. Commercial codfish extract was tested at six 3.5-fold dilutions, starting with 1:10 of the stock solution. Measurements with fresh codfish were performed in 12 dilutions with a dilution factor of 3.5. Twenty-five microlitres of heparinized whole blood was applied to allergen-coated microtitre wells (Reference Laboratory, Copenhagen, Denmark) and incubated with 25 μ l of allergen or anti-IgE for 1 h at 37°C. Thereafter the plates were rinsed and interfering substances were removed. Finally, histamine bound to the microfibrils was measured spectrophotofluorometrically [16,17]. A histamine release of 20 ng/ml whole blood or more was considered positive. Subjects with non-responding basophils, i.e. a histamine release of less than 20 ng/ml following anti-IgE challenge, were classified as inconclusive.

SDS-PAGE

In brief, SDS-PAGE was performed as previously described [18] with 16% separation gels and 5% stacking gels in a vertical electrophoresis chamber. Samples were diluted in a sample buffer containing 5% mercaptoethanol and boiled for 5 min. An amount of 0.9 mg of codfish extract in 30 μ l was loaded onto 0.5 cm of gel. Molecular weights (mol. wt.) were estimated using prestained molecular weight markers (Amersham, 14.3–200 kDa). Electrophoresis was performed at 13 mA overnight.

The gels were either stained by Coomassie for protein determinations or electroblotted for subsequent determination of IgE-binding.

Immunoblot (IB): an amount of 0.9 mg in 30 μ l was applied pr 0.5 cm of gel. A radioactive molecular weight marker (CFA 756, Amersham) was used for estimation of molecular weight in the immunoblot experiments.

Electroblotting and detection of specific IgE

The codfish proteins were transferred from the polyacrylamide gels onto nitrocellulose paper (pore size 0.2 μ m, Schleicher & Schuell, Germany) by a semidry electroblotter (JKA-Biotech, Denmark) at 0.8 mA/cm² for 1½ h in a discontinuous buffer system at room temperature (anodic buffers pH 10.4 and cathodic buffer pH 9.4, containing 20% methanol).

Nitrocellulose was cut into strips of 0.5 cm and blocked in Tris-buffered saline containing 3% w/v powdered milk and 1.5% Tween 20 (blocking solution), for 30 min at room temperature.

Subsequently strips were incubated overnight with 1 ml of serum diluted 1:10 (1 patient's serum reacted very strongly, and had to be diluted 1:200) in blocking solution. Strips were washed (0.5% Tween 20 in TBS buffer, pH 7.4) and bound antigen-specific IgE was detected by ¹²⁵I-labelled rabbit anti-human IgE (Pharmacia, $\approx$ 200 000 cpm) diluted 1:12 in blocking solution, overnight. All incubation steps were performed at room temperature on a rocking platform.

Control strips were tested with a pool of serum from normal patients and a buffer control, respectively. These controls were invariably negative. The nitrocellulose strips were washed, air-dried, and exposed to hyperfilm (Amersham, MP, RPN. 6) for 3, 7, and 14 days at –80°C.

Calculations and statistics

Sensitivity (SE) and specificity (SP) of the various tests were calculated for the commercial as well as the fresh extracts according to guidelines from the Workshop on Experimental Methodology [2]. Calculations of specificity were based on all codfish-tolerants in one group. In this study a small and highly selected group of patients participated, so SE and SP represent the ability of the tests to discriminate between DBPCFC-positives and codfish-tolerant subjects.

An optimal cut-off level was determined, i.e. the cut-off in units/dilution giving maximum improvements of diagnostic accuracy, which means increasing SP without decreasing SE, or improving SE without losing SP.

For comparison of results of IgE-tests in DBPCFC-positives compared with DBPCFC-negatives, the Mann-Whitney test was used. Correlations of specific IgE tests were based on Spearman's test.

Results

Specific IgE — commercial tests

The codfish-specific serum IgE levels were significantly higher ($P < 0.05$) in the challenge positive group compared with the codfish-tolerant control subjects in all assays performed. The values of codfish-specific IgE are shown in Fig. 1.

The number of positive results in PHA, CAP and ML are shown in Table 2. All three tests correctly identified the eight clinical codfish allergics as positive, i.e. a SE of 1.00. False positive reactions were seen in three codfish-tolerant control subjects using PHA (one DBPCFC-negative and two with atopic dermatitis, all having negative SPT), in four when using CAP (two DBPCFC-negatives of which one had a positive SPT and the other had positive PHA, and the same two with atopic dermatitis), while no false positive reactions were registered using ML.

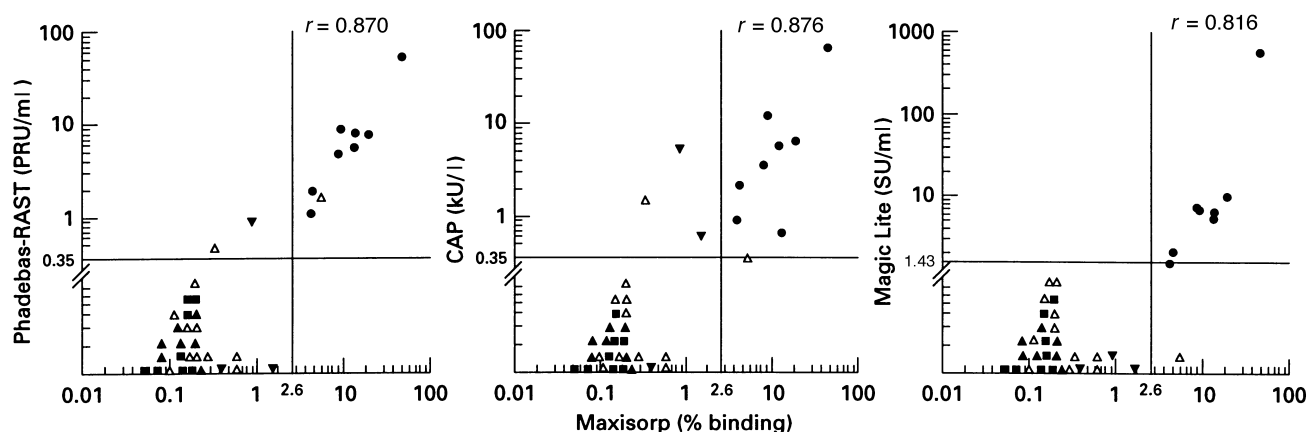


Fig. 1. Correlation between specific IgE analyses performed with commercial codfish extract and freshly prepared extract of codfish (MAXI). Cut-off suggested by the manufacturers (class 1) and the cut-off level of MAXI (% bound radioactivity) are indicated. ● = DBPCFC-positive; ▼ = DBPCFC-negative; ■ = non-atopics; ▲ = pollen allergics; △ = atopic dermatitis.

Changing the cut-off level in PHA from ≥ 0.35 PRU/mL to ≥ 1.00 PRU/mL increased SP of the test from 0.90 to 0.97. The same was true for CAP (0.87–0.93) by increasing cut-off from ≥ 0.35 kU/l to ≥ 0.67 kU/l, whereas the SP of ML was already optimal (1.00) at the manufacturer's suggested cut-off level of >1.43 SU/mL, i.e. correctly identifying DBPCFC-positives in control subjects in all

cases. Thus, by optimizing the cut-off level, improvement in diagnosis was achieved, i.e. improved diagnostic accuracy.

HR — commercial extract

Figure 2 shows SE and SP of the HR-test. Two DBPCFC-positives had non-responding basophils (i.e. basophils

Table 2. No. of positives in specific IgE analyses. Specific IgE assays performed in all clinical codfish allergies (DBPCFC-positives), and in codfish-tolerant control subjects. Number of positive results using the cut-off level as suggested by the manufacturers, compared with positive results if using the optimal cut-off level. The optimal cut-off is the maximum improvement in diagnosing, which means reducing the number of false positives without losing SE. In MAXI, no suggested cut-off is recommended

	DBPCFC+ (n = 8)		DBPCFC– (n = 3)		Non-atopic (n = 8)		Pollen all. (n = 7)		AD (n = 12)	
	Suggested	Optimal	Suggested	Optimal	Suggested	Optimal	Suggested	Optimal	Suggested	Optimal
Phadebas	8	8	1	0	0	0	0	0	2	1
CAP	8	8	2	1	0	0	0	0	2	1
Magic Lite	8	8	0	0	0	0	0	0	0	0
Maxisorp	–	8	–	0	–	0	–	0	–	1

	Cut-off	
	Suggested	Optimal
Phadebas (PRU/mL)	≥ 0.35	≥ 1.0
CAP (kU/l)	≥ 0.35	≥ 0.67
Magic Lite (SU/mL)	> 1.43	> 1.43
Maxisorp (% binding)	–	≥ 2.6

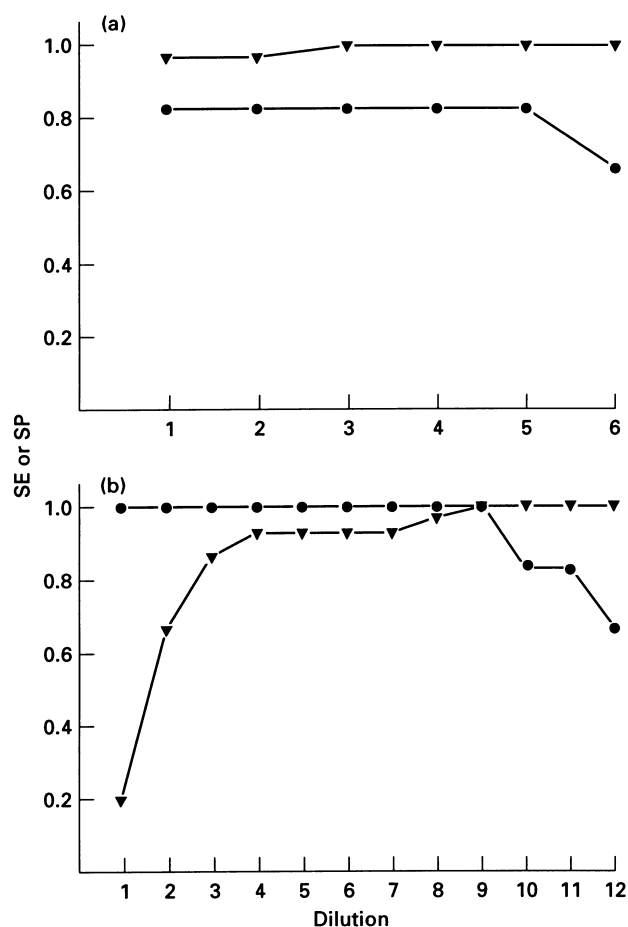


Fig. 2. Histamine release with commercial and fresh codfish extracts. Titration of the allergen preparations. SE, sensitivity: the number of true positives of all with a positive diagnosis. SP, specificity: the number of true negatives of all without codfish allergy. Two DBPCFC-positives were inconclusive. (a) Commercial extract: dilution 1 = 1:10; dilutions 2–6 = 3.5-fold dilutions, i.e. $1:10 \times 3.5$, $1:10 \times 3.5^2$, etc. (b) Fresh extract: dilution 1 = 1:20; dilutions 2–12 = 3.5-fold dilutions, i.e. $1:20 \times 3.5$, $1:20 \times 3.5^2$, etc. ● = SE; ▼ = SP.

releasing < 20 ng histamine/ml following anti-IgE challenge) and were excluded from the calculations.

No improvement in SE (0.83) could be obtained by diluting the commercial codfish extract, and thereby varying the cut-off, but false positive reactions were reduced (improvement in SP: 0.97–1.00) by a proper dilution ($1:10 \times 3.5^2$) of the allergen.

Specific IgE — fresh extract

To improve SP of IgE-tests and SE of HR, a fresh, crude codfish extract was tested. When used in the in-house specific-IgE test, MAXI, no advantage was obtained,

compared with the outcome of commercial tests (SE 1.00, SP 0.97). Thus, the rank of diagnostic accuracy of specific IgE tests (shown in Table 2) is ML > PHA/MAXI > CAP.

Comparing the outcome of MAXI with the commercial specific IgE-analyses, using the manufacturers' cut-off, correlations of $r = 0.816$ – 0.876 were seen.

One control subject with atopic dermatitis and high total IgE, who was a clinical codfish-tolerant (having a positive CAP and PHA), showed a consistently high response in MAXI (reproduced).

HR — fresh extract

When using freshly prepared codfish extract a SE of 1.00 could be obtained together with a maximum SP of 1.00 as well (Fig. 2). The optimal dilution of the fresh, crude extract was number 9, i.e. 0.002 mg/ml.

SDS-PAGE

In order to further characterize the fresh extract, this was subjected to analyses by SDS-PAGE and IB. In Coomassie stained SDS-PAGE with fresh raw codfish extract, proteins were separated into numerous bands with molecular weights ranging from 200 to <14 kDa, Fig. 3a. The protein profile and intensity were demonstrated (≈ 29 bands, of which 12 were prominent), and a band migrating at 12–13 kDa corresponding to the major allergen, Gad cI, was seen. The observed molecular weights for the other most prominent bands were ≈ 16 , 18, 21, 27, 31, 35, 37, 43, 52, 90, 150 kDa.

Immunoblotting

SDS-PAGE-separated codfish proteins were probed with sera from all patients and control subjects and were examined individually for specific IgE binding, Fig. 3b (IB-results of all control subjects not shown).

All eight DBPCFC-positives showed strong binding to the band at about 12–13 kDa, whereas no significant binding was observed for the codfish-tolerant control subjects. Two of the eight DBPCFC-positives showed binding mainly to that band, whereas the remaining six out of eight also bound several other proteins. Ten of the 30 codfish-tolerant control subjects (three DBPCFC-negatives and seven with atopic dermatitis and high total IgE) bound in the 18–200 kDa area (one DBPCFC-negative showing very weak binding). No binding was observed for the non-atopics or pollen allergics.

The observed molecular weights for other allergens were 18, 22, 23, 26, 31, 33, 35, 37, 43, 49, 56, 69, 81, >97.4 kDa. The differences were all mainly observed in the intensity of staining the individual bands for minor allergens. No bands were observed for buffer.

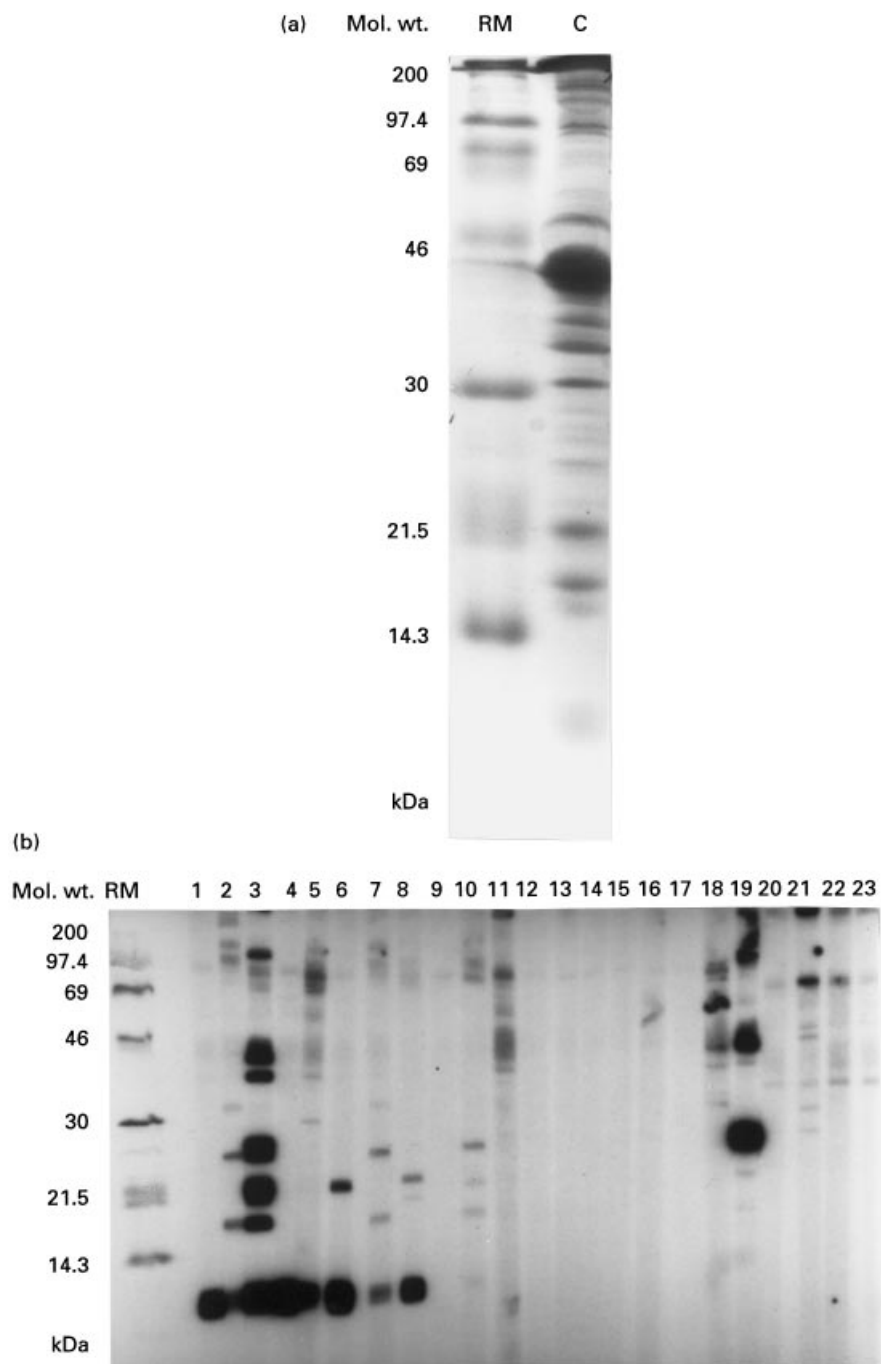


Fig. 3. (a) Coomassie stained 16% separation SDS-polyacrylamide gel showing the protein pattern of freshly prepared codfish extract. Loading dose: 0.9 mg/30 μ l. Mol.wt.: molecular weight standard in kiloDaltons. Sample buffer containing mercaptoethanol. (b) IgE binding pattern in freshly prepared codfish extract using sera from DBPCFC-positives and codfish-tolerant control subjects. Load: 0.9 mg/0.5 cm. Sera was diluted 1:10, pt. 3: 1:200. Bound IgE was detected with ^{125} I-labeled rabbit antihuman IgE: approximately 200 000 cpm/strip. Blots were exposed to X-ray films for 7 days. Mol.wt.: radioactive molecular weight marker (RM) (Da). 1–8: DBPCFC-positives. 9–23: codfish-tolerant control subjects (9–11, DBPCFC-negatives; 12–14, non-atopics; 15–17, pollen allergics; 18–23, atopic dermatitis, 5 of these with total IgE > 2000 kIU/l).

The main finding was the strong and specific binding of IgE to the 12–13 kDa band, positive in all clinically codfish-allergic patients, SE and SP of 1.00, respectively. Binding to other allergens was seen in all DBPCFC-positives and in 33% of all control subjects.

Discussion

We compared histamine release and levels of specific serum IgE measured by various methods using fresh and commercial codfish preparations with the outcome of DBPCFC (performed with fresh codfish extract) in a small group of adults with a convincing history of immediate allergic reactions to codfish. Our interpretations should be tempered by the fact that the actual prevalence of fish allergy together with the rigorous criteria and the very cumbersome protocol, making numerous appearances at the clinic necessary, forced us to work with a limited number of patients.

Before evaluating a test, the disease in question has to be established by diagnostic methods of proven value — any new test must be evaluated against a 'gold standard' [3,19]. In spite of rigorous criteria for having a convincing history, only 8/11 were classified as codfish allergics, using DBPCFC.

When calculating SP it is essential to describe the population involved. Using normal non-atopic control subjects raises the possibility of obtaining almost any SP of a test. We therefore used a control group resembling that which is seen in our clinic, and thereby had the relevant tests performed: 30 subjects, 10% having a positive case history but being codfish-tolerant according to DBPCFC, 40% having atopic dermatitis, 27% non-atopics, and 23% with pollen allergy. This gives rise to a more realistic estimation of SP.

Specific IgE

Overall, a very high SE was found in all assays, whereas minimal differences in SP were seen: ML > PHA > CAP. This makes PHA and CAP acceptable for screening for IgE, but not sufficient for diagnosing, even by using the optimal cut-off levels. Compared with the outcome of DBPCFC some false positive reactions were found in CAP and PHA, but not in ML. However, the diagnostic accuracy of ML was optimal, since ML correctly identified DBPCFC-positives and control subjects in all cases, which might indicate that the codfish extract used in ML mainly contains relevant proteins (i.e. Gad cI). Furthermore, Fig. 1 shows that it is possible to optimize the cut-off level suggested by the manufacturers and consequently improve the diagnostic accuracy of PHA and CAP.

Extracts of fresh food have been shown to improve diagnostic accuracy [5,20] compared with commercially available extracts. Only a few false positive results were

seen with commercial extracts in CAP and PHA in our study, and no improvement in diagnosis was achieved by using self-prepared codfish extract. One false positive reaction was seen in MAXI, who was the same false positively reacting person found in CAP and PHA. This control subject (having atopic dermatitis and a total IgE >2000 kIU/l) had been openly challenged according to another protocol. Furthermore, he reacted in IB, especially to the 26 and 43 kDa bands.

Research findings have revealed a concordance between challenge results (single-blind challenges with fish), RAST of 93%, a SE of 91%, and a SP of 94% [21], and in a later study by Aas [11] using a purified codfish protein a 100% correlation was found between a positive RAST and the presence of clinical allergy to cod in children. We found identical results in this study (DBPCFC and a specific-IgE test: SE 100%, SP 87–100%, concordance 90–100%).

HR

The role of HR in diagnosing food-allergic subjects has been investigated in different studies with various allergens and different study designs [4,5,12,22,23].

We have previously compared HR, SPT and PHA with a commercial codfish extract and found HR less sensitive [12]. This might be due to a suboptimal extract used in HR, since in this study we could increase SE and diagnostic accuracy by using a fresh extract.

In the study we evaluated the utility of this test in eight clinical codfish allergics (two of these (25%) were non-responders and were excluded from calculations). HR with commercial extract was positive in five out of six DBPCFC-positives (83%) and in one (3%) of the control subjects.

False positive reactions were seen amongst the control subjects at high allergen concentrations, but this factor could be eliminated by dilution of the commercial allergen. By doing this, a SE/SP of 0.83/1.00, respectively, could be obtained. It was a problem that two DBPCFC-positives were non-responders, and if these had been included as false negatives, the SE would have dropped to 0.63.

When the fresh codfish extract was used for HR, the SE was improved from five out of six (=0.83) using commercial extracts to 1.00, without losing SP (if the non-responders are regarded as false negatives, SE is improved from five out of eight (=0.63, commercial) to six out of eight (=0.75, fresh)).

SDS-PAGE & immunoblotting

SDS-PAGE and immunoblotting were applied in order to ensure allergen quality, but the data obtained might also be used in the search for an explanation of the problems about specificity of the tests. SDS-PAGE and immunoblotting

revealed that the major allergen of codfish, Gad cI, could be used for diagnosing, and this should be further investigated. Gad cI, is composed of 113 amino acid residues in a single polypeptide chain with a molecular weight of 12 328 Da [23]. The composition of fresh allergenic codfish extract was shown by using SDS-PAGE and immunoblotting, and a prominent band migrating at ≈ 12 –13 kDa was found, believed to represent a protein fraction analogous to Gad cI.

Sera from clinical codfish allergics contains specific IgE strongly directed against the immunodominant protein, presumably Gad cI, whereas no significant binding was observed for the codfish-tolerant control subjects, which means SE and SP of 1.00. Binding to proteins with a higher molecular weight was seen in control subjects, especially in the fish-tolerant person reacting in specific IgE tests as mentioned above.

Repeated recognition of codfish protein bands by the DBPCFC-positives' sera, suggests that there is a certain key allergen to which clinical codfish allergics will respond. However, immunoblotting is a qualitative method of allergen characterization, and the intensity of the radiostaining of allergens depends on the concentration of IgE in the serum, the strength/affinity of the anti-IgE label used, and the duration of autoradiography. It cannot be excluded that some of the recognized reactions of high intensity may contain more than one band.

In this study we investigated a small and highly selected group of patients. They all underwent DBPCFC with fresh codfish, but it is important to consider that the discrimination between DBPCFC-positive and -negative depends on how sensitive the patient is — i.e. how small a dose of codfish he will react to. Since the maximum dose used in this study was 6.7 g, it cannot be excluded that some of the DBPCFC-negatives would have reacted to higher amounts, but the doses used for challenging were selected according to the amount reported to elicit adverse allergic reactions.

Conclusion

Our study suggests that *in vitro* assays can identify patients who are clinically allergic to codfish. Fresh extract could improve the outcome of HR, whilst no advantage was seen using this in specific IgE tests. Overall, these tests are acceptable for screening for codfish allergy, but only ML or a specific and strong binding of IgE to the 12–13 kDa protein are sufficient for establishing the diagnosis, when using DBPCFC as the 'true diagnosis'.

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References

- 1 Prausnitz C, Küstner H. Studien über die Ueberempfindlichkeit. *Centralbl f Bakt* 1921; 86:160–9.
- 2 Metcalfe DD, Sampson HA. Workshop on Experimental Methodology for Clinical Studies of Adverse Reactions to Foods and Food Additives. *J Allergy Clin Immunol* 1990; 86:421–42.
- 3 Bruijnzeel-Koomen C, Ortolani C, Aas K et al. Position paper. Adverse reactions to food. *Allergy* 1995; 50:623–35.
- 4 Bernstein M, Day JH, Welsh A. Double-blind food challenge in the diagnosis of food sensitivity of the adult. *J Allergy Clin Immunol* 1982; 70:205–10.
- 5 Bock SA, Lee WY, Remigio L, Holst A, May CD. Appraisal of skin tests with food extracts for diagnosis of food hypersensitivity. *Clin Allergy* 1978; 8:559–64.
- 6 Bindslev-Jensen C, Stahl Skov P, Madsen F, Poulsen LK. Food allergy and food intolerance — what is the difference? *Ann Allergy* 1994; 72:317–20.
- 7 Sampson HA, Albergo R. Comparison of results of skin tests, RAST, and double-blind, placebo-controlled food challenges in children with atopic dermatitis. *J Allergy Clin Immunol* 1984; 74:26–33.
- 8 Aas K. The diagnosis of hypersensitivity to ingested foods. *Clin Allergy* 1978; 8:39–50.
- 9 de Martino M, Novembre E, Galli L et al. Allergy to different fish species in cod-allergic children: *in vivo* and *in vitro* studies. *J Allergy Clin Immunol* 1990; 86:909–14.
- 10 Bernhisel-Broadbent J, Scanlon SM, Sampson HA. Fish hypersensitivity. I: *In vitro* and oral challenge results in fish-allergic patients. *J Allergy Clin Immunol* 1992; 89:730–7.
- 11 Aas K, Lundkvist U. The radioallergosorbent test with a purified allergen from codfish. *Clin Allergy* 1973; 3:255–61.
- 12 Hansen TK, Bindslev-Jensen C. Codfish allergy in adults. Identification and diagnosis. *Allergy* 1992; 47:610–7.
- 13 Dreborg S, ed. Skin tests used in type I allergy testing. Position Paper. Sub-committee on Skin Tests of the European Academy of Allergology and Clinical Immunology. *Allergy* 1989; 44, suppl. 10:22–30.
- 14 Hanifin JM, Rajka G. Diagnostic features of atopic dermatitis. *Acta Derm Venereol* 1980; suppl. 92:44–7.
- 15 Poulsen LK, Pedersen MF, Malling H-J, Søndergaard I, Weeke B. Maxisorp RAST. A sensitive method for detection of antigen-specific human IgE in culture fluids. *Allergy* 1989; 44:173–80.
- 16 Nolte H, Storm K, Schiøtz PO. Diagnostic value of a glass fibre-based histamine analysis for allergy testing in children. *Allergy* 1990; 45:213–23.
- 17 Stergaard PA, Ebbesen F, Nolte H, Stahl Skov P. Basophil histamine release in the diagnosis of house dust mite and dander allergy of asthmatic children. *Allergy* 1990; 45:231–5.
- 18 Jarolim E, Poulsen LK, Stadler BM et al. A long-term follow-up study of hyposensitization with immunoblotting. *J Allergy Clin Immunol* 1990; 85:996–1004.
- 19 Björkstén B. *In vitro* diagnostic methods in the evaluation of food hypersensitivity. In Metcalfe DD, Sampson HA, Simon RA, eds. *Food Allergy. Adverse Reactions to Foods and Food Additives*. Oxford: Blackwell Science, 1991:67–80.

- 20 Aas K. Studies of hypersensitivity to fish. *Int Arch Allergy* 1966; 30:257–67.
- 21 Aas K, Johansson SGO. The radioallergosorbent test in the in vitro diagnosis of multiple reagenic allergy. *J Allergy Clin Immunol* 1971; 48:134–42.
- 22 Pastorello A, Stocchi L, Bigi A et al. Value and limits of diagnostic tests in food hypersensitivity. *Allergy* 1989; 44, suppl. 9:151–8.
- 23 Norgaard A, Skov PS, Bindslev-Jensen C. Egg and milk allergy in adults: comparison between fresh foods and commercial allergen extracts in skin prick test and histamine release from basophils. *Clin Exp Allergy* 1992; 22:940–7.
- 24 Elsayed S, Bennich H. The primary structure of allergen m from cod. *Scand J Immunol* 1975; 4:203–8.