Allergy to fish parvalbumins: Studies on the cross-reactivity of allergens from 9 commonly consumed fish

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Background: Fish-hypersensitive patients can probably tolerate some fish species while being allergic to others.
Objective: To determine the allergenic cross-reactivity between 9 commonly edible fish: cod, salmon, pollack, mackerel, tuna, herring, wolffish, halibut, and flounder.
Methods: Sera from 10 patients allergic to fish and rabbit antisera against 3 parvalbumins (Gad c 1, Sal s 1, and The c 1) were used. Cross-reactivity was investigated by SDS/PAGE and IgE immunoblotting, IgG ELISA, IgE ELISA inhibition, and skin prick test (SPT).
Results: Cod (Gad c 1), salmon (Sal s 1), pollack (The c 1), herring, and wolffish share antigenic and allergenic determinants as shown by immunoblots and IgE ELISA, whereas halibut, flounder, tuna, and mackerel displayed lowest cross-reactivities. The highest mean IgE ELISA inhibition percent of 10 sera was obtained by Gad c 1, followed by The c 1, herring, Sal s 1, wolffish, halibut, flounder, tuna, and mackerel with the least inhibition. Nine of the 10 patients showed positive SPT to cod, salmon, and pollack; 8 patients reacted to recombinant (r) Sal s 1. Positive SPTs to rGad c 1 and rThe c 1 were demonstrated in 1 patient.
Conclusion: Gad c 1, Sal s 1, The c 1, herring, and wolffish contained the most potent cross-reacting allergens, whereas halibut, flounder, tuna, and mackerel were the least allergenic in the current study. The latter could probably be tolerated by some of the tested patients. (J Allergy Clin Immunol 2005;116:1314-20.)

***Key words:** Fish allergy, cross-reactivity, parvalbumin, recombinant allergen, cod, pollack, salmon*

Abbreviations used

DBPCFC:	Double-blind, placebo-controlled food challenge
r:	Recombinant
SPT:	Skin prick test

Fish plays an important role in the human food, providing a valuable source of highly assimilated proteins, but it is also among the most common causes of food allergy.[1-3] Atopic allergy to fish is particularly common in children and young adults. The clinical symptoms related to fish allergy might be manifested in a variety of symptoms (eg, urticaria, allergic contact dermatitis, rhino-conjunctivitis, asthma, oral allergy syndrome, diarrhea, or anaphylaxis). Fish hypersensitivity is frequently encountered in coastal countries like Norway, where considerable numbers of the population work in the fish industry, and fish is constantly consumed. Exposure to fish allergens can be through inhalation of airborne allergens during outdoor drying, skin contact while filleting and cooking fish, or ingestion of fish meals. Fish allergy has been reported to occur in about 0.1% of the Norwegian population.[1] Most of the patients allergic to fish do not tolerate cod; therefore, this is usually used as reference to which other fish allergens are related. Codfish hypersensitivity has been extensively studied, and the major allergen Gad c 1 (allergen M) has been found to be a parvalbumin.[4-8] Fish muscle parvalbumin is a stable acidic Ca^{2+} binding protein (12 kd), resistant to heat, chemical denaturation, and proteolytic enzymes.[9-11] Parvalbumins are present in high amounts in white muscles of lower vertebrates[12] and in lower amounts in fast twitch muscles of higher vertebrates.[13] It has been demonstrated that parvalbumin is present in white muscle of many fish species; thus, cross-reactivity among different fish species might exist.[1,3,11,14-17] However, patients allergic to codfish can ingest some other species without risk of allergic symptoms, as shown by some previously reported studies.[15,16,18-20] In the current study, the cross-reactivity between Gad c 1 parvalbumin and 8 of the most commonly edible fish species in Norway was examined by several *in vitro* assays and skin prick test (SPT).

METHODS

Patients with fish allergy and controls

Twelve patients were recruited from the ambulant patients routinely examined at the Laboratory of Clinical Biochemistry and

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Supported by a grant from the Western Norway Regional Health Authority (Helse Vest).
Received for publication November 16, 2004; revised July 4, 2005; accepted for publication July 26, 2005.
Available online October 4, 2005.
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0091-6749/$30.00

doi:10.1016/j.jaci.2005.07.033

TABLE I. Summary of clinical histories and laboratory data*

Patient no.	Age	Sex	Symptoms	Total serum IgE	Specific IgE CAP FEIA, kU/L Gad c 1	Sal s 1	Tuna	Herring	Mackerel
1	43	F	Anaphylaxis	56	10.30	12.90	4.42	20.40	2.88
2	33	F	Anaphylaxis	121	2.52	1.33	0.38	9.86	<0.35
3	42	F	Anaphylaxis	159	0.52	0.50	<0.35	0.95	<0.35
4	37	F	Anaphylaxis	67	1.39	<0.35	<0.35	<0.35	<0.35
5	27	M	Oral allergy syndrome, contact urticaria	79	13.80	22.20	7.45	19.00	4.29
7	25	M	Anaphylaxis	134	0.95	0.79	0.45	1.55	<0.35
8	21	F	Oral allergy syndrome	487	13.20	8.75	4.97	14.10	3.56
9	55	F	Anaphylaxis, oral allergy syndrome, contact urticaria	1712	>100	78.20	25.20	>100	17.10
11	53	F	Anaphylaxis, contact urticaria	86	4.46	10.90	0.95	7.89	1.43
12	30	F	Abdominal pain, flushing, dyspnea	308	5.18	3.04	1.08	6.50	0.85

*Specific IgE CAP-FEIA expressed in kU/L. All of the patients have IgE-mediated allergy.

the Centre for Occupational and Environmental Allergy, Haukeland University Hospital, Bergen, Norway. Two of them were excluded from this report because they did not show IgE-mediated allergy to fish. Ten patients, 8 women and 2 men, age 21 to 55 years, had histories of generalized or anaphylactic reactions after intake of cod on at least 2 occasions. The clinical data supporting allergy and serum total and specific IgE values as well as the CAP-FEIA classes are given in Table I. Seven have histories of generalized anaphylaxis after ingestion or direct contact with fish fillet and cooking fish. Five patients have variable gastrointestinal tract manifestations after fish meals. Ten patients have relatively high values of serum total (mean 312 kU/L) and specific IgE for cod and salmon. One patient (#9) strongly reacted to tuna and mackerel; 3 (#1, 5, and 9) strongly reacted to herring. Patient #9 has very high total and specific IgE against 5 fish species and intensive IgE-mediated anaphylactic response to most of the fish sorts tested. No double-blind, placebo-controlled food challenge (DBPCFC) could possibly be performed on the tested population because of an inherent risk of anaphylaxis. Ten control subjects (tolerating fish) were included. Informed consent was obtained from each volunteer, and the study was approved by the Regional Committee for Medical Research Ethics in Western Norway (REK Vest).

Rabbit IgG

Rabbit polyclonal antibodies against Gad c 1, Sal s 1, and The c 1 parvalbumins were usually raised in rabbits at the University of Bergen, Animal House (Vivarium), by the methods described previously.[7,21,22]

Preparation of fish extracts and fish parvalbumins

Atlantic cod (*Gadus morhua*), Atlantic salmon (*Salmo salar*), Atlantic mackerel (*Scomber scombrus*), tuna (*Thunus albacares*), herring (*Clupea harengus*), wolffish (*Anarhichas sp*), halibut (*Hippoglossus hippoglossus*), and flounder (*Platichthys flesus*) were purchased from the fish market in Bergen. Alaska pollack (*Theragra chalcogramma*) was purchased as frozen fillet produced by FRoSTA AG, Bremenhaven, Germany. Fish extracts and parvalbumins were obtained by using update laboratory instrumentation and methodology by the methods classically described elsewhere.[4,23]

Recombinant fish parvalbumins

The production of the recombinant (r) parvalbumins rGad c 1, rSal s 1, and rThe c 1 has been described previously.[21,24,25]

IgG ELISA

ELISA was performed by using highly purified polyclonal rabbit IgG.[22] Briefly, 96-well, flexible round bottom microtiter plates (Dynatech Laboratories Inc, Chantilly, Va) were coated with 0.5 μg Gad c 1 in 100 μL buffer, pH 9.5. Coating was performed overnight at 4°C. This was followed by washing (Tris-Tween buffer, pH 7.4), and purified polyclonal IgG against Gad c 1, Sal s 1, and The c 1 (diluted 1.10^{-4}) was added and incubated for 2 hours at room temperature. After another washing, antirabbit IgG alkaline phosphatase conjugate (Sigma Chemical Co, St Louis, Mo) was used for incubation for 2 hours. Finally, after another wash, the color was developed by incubation with 100 μL/well Tris buffer pH 9.5, containing 1 mg/mL *p*-nitrophenylphosphate (Sigma). Absorbance was read at $\lambda = 405$ nm after 10 minutes.

IgE ELISA inhibition

IgE ELISA inhibition was performed as described previously.[21,24,25] Briefly, plates were coated with 1 μg Gad c 1 (100 μL/100 mmol/L sodium bicarbonate buffer, pH 9.6). Patients' sera (50 μL) were inhibited by incubation with 100 μg/100 μL Gad c 1, Sal s 1, and The c 1 parvalbumins, or purified allergen of halibut, mackerel, herring, wolffish, flounder, tuna, and rGad c 1, rSal s 1, and rThe c 1.

SDS-PAGE and specific IgG/IgE–immunoblotting

Fish extracts were separated by SDS-PAGE. The samples along with molecular weight standards were resolved in a 15% separating gel at 200 V. Proteins were visualized by Coomassie brilliant blue R-250 staining (Sigma). For immunoblot analyses, proteins were transferred onto nitrocellulose membranes (0.45 μm) using a minitrans-blot cell (BIO-RAD Laboratories, Richmond, Calif) for 1 hour at 100 V. Immunodetection of cross-reactivities between allergens was performed with the serum pool of patients allergic to fish or polyclonal rabbit IgG. After antibody binding, the color reaction was developed with SIGMA FAST BCIP/NBT tablets (Sigma).[21,25]

SPT

SPTs were performed in duplicate according to the guidelines of the European Academy of Allergology and Clinical Immunology Subcommittee on skin tests[26] with native and recombinant Gad c 1, Sal s 1, and The c 1 (1 mg/mL), dissolved in sterile physiological saline solution. Reactions were recorded after 15 minutes by

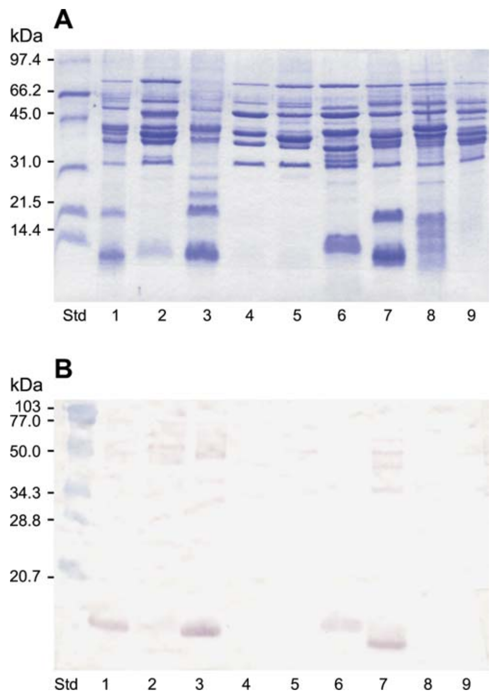

FIG 1. SDS-PAGE **(A)** and immunoblotting **(B)** of fish allergens. The blot was probed with a serum pool of patients allergic to fish. *Std,* Standard molecular weight; *1,* Gad c 1; *2,* Sal s 1; *3,* The c 1; *4,* halibut; *5,* mackerel; *6,* herring; *7,* wolffish; *8,* flounder; *9,* tuna.

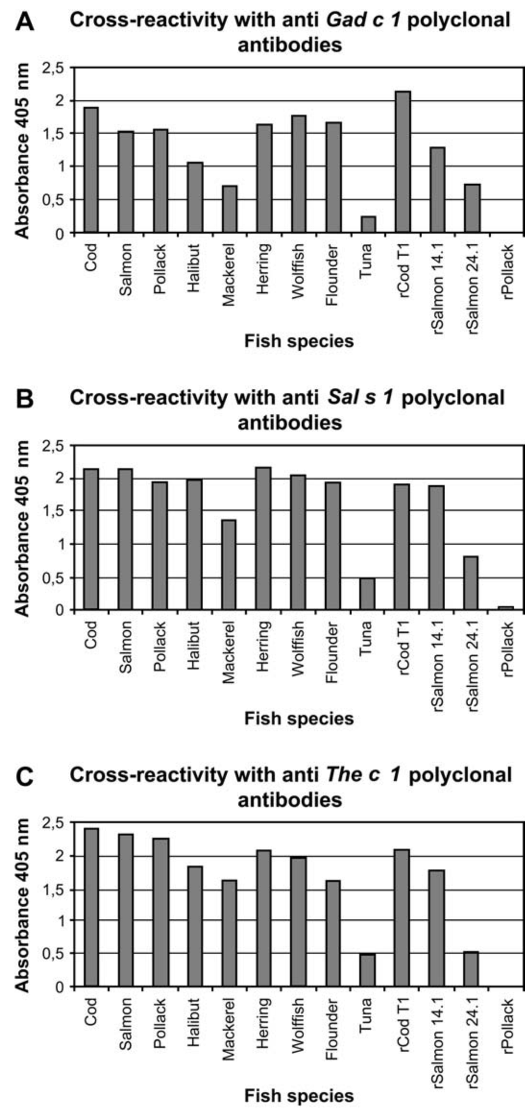

FIG 2. IgG ELISA using antibodies against Gad c 1 **(A)**, Sal s 1 **(B)**, and The c 1 **(C)**. Cross-reactivities of different fish species as well as the recombinant parvalbumins are shown.

measuring the mean of the 2 perpendicular diameters of the duplicate wheals and transferring the ballpoint pen–marked wheal reaction by single adhesive tape to paper. The mean diameter of the wheal was calculated following the formula: (D1 + D2)/2. A mean wheal diameter of 3 mm larger than that of the negative control (0.9% saline solution) was considered positive. Histamine chloride (10 mg/mL) was used as positive control. The SPT was performed by 2 nurses and yielded a mean histamine wheal diameter of 4.88 mm (mean of the 10 duplicates). The coefficient of variation (CV = SD × 100/mean of wheal diameters tested with histamine) between duplicates was 0.155.

RESULTS

Identification of IgE-binding proteins

Initially, fish purified allergen extracts were characterized by SDS-PAGE and immunoblotting using a serum pool of patients allergic to fish. The extract patterns are presented in Fig 1, *A*. The proteins were resolved into many bands (molecular weights range 12-97 kd). An intensive colored band in cod, salmon, pollack, and wolffish extracts was observed at approximately 12 kd, corresponding to parvalbumin (allergen M, Gad c 1), whereas the parvalbumin band in herring extract was localized at 14 kd. Halibut and mackerel showed weak bands at approximately 12 kd, whereas the tuna parvalbumin band was almost invisible. Immunoblotting revealed clearly stained bands for cod, salmon, pollack, herring, wolffish, and flounder extracts. Three fish (halibut, mackerel, and tuna), which showed very weak or invisible parvalbumin bands in SDS-PAGE (Fig 1, *A*), gave no IgE-binding for samples #4, 5, 8, and 9 (Fig 1, *B*). Weakly stained IgE-binding proteins were shown at the high molecular weight region; figures for IgG-immunoblotting are not shown.

Identification of IgG-binding proteins

To ascertain the antigenicity of the different fish extracts, immunoblots were also performed by using rabbit polyclonal antibodies against Gad c 1, Sal s 1, and The c 1 parvalbumins. These recognized Gad c 1, Sal s 1, The c 1, and herring and wolffish parvalbumins in a pattern similar to that of the IgE runs shown in Fig 1, *A and B*.

IgG ELISA

Polyclonal IgG against Gad c 1, Sal s 1, and The c 1 recognized all of the native parvalbumins and fish extracts

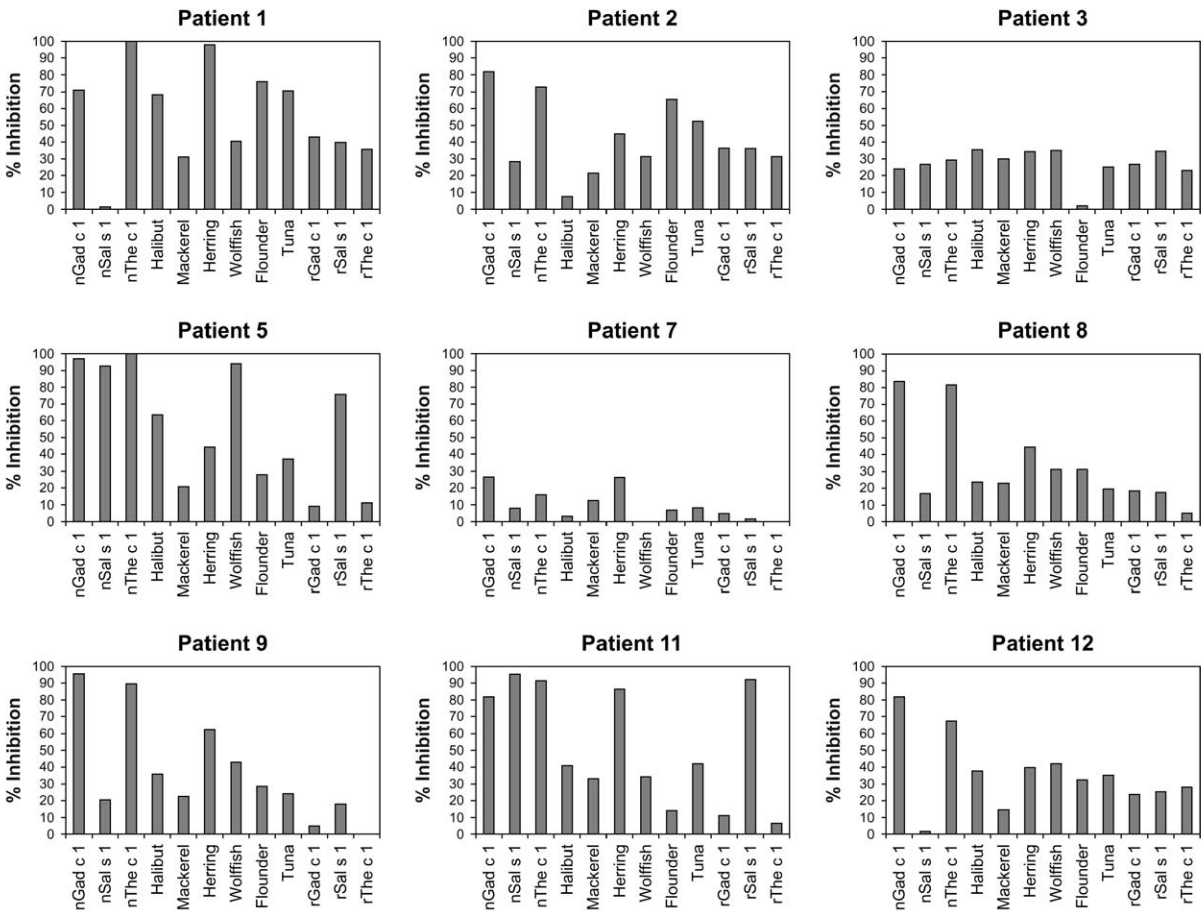

FIG 3. IgE ELISA inhibition. All of the 10 sera were inhibited by native (n) Gad c 1, nSal s 1, and nThe c 1 and extracts of halibut, mackerel, herring, wolffish, flounder, and tuna as well as by rGad c 1, rSal s 1, and rThe c 1.

at different grades. Three of the recombinant parvalbumins (rGad c 1, rSal s 1 14.1, and rSal s 1 24.1) were similarly recognized (Fig 2, *A-C*). Recombinant The c 1 was not recognized by the 3 used sera. The mean absorbances (λ = 405 nm) of IgG ELISA of those sera were as follows: for Gad c 1, IgG ranging from 0.250 (tuna) to 1.888 (cod); for Sal s 1, IgG ranging from 0.477 (tuna) to 2.171 (herring); and for The c 1, IgG ranging from 0.481 (tuna) to 2.414 (cod). Gad c 1, Sal s 1, and The c 1 were the most potent of homologous antibodies. High affinity was found for rGad c 1 T1 using homologous antibodies (Fig 2, *A*). Except for mackerel, tuna, and rThe c 1, all of the other sorts have high cross-reactivity with salmon (Fig 2, *B*). Similarly, all of the fish sorts except tuna and rSal s 1 24.1 and rThe c 1 had high cross-reactions to pollack IgG antibodies (Fig 2, *C*). Tuna and mackerel were the least cross-reacting fish.

IgE ELISA inhibition

For further determination of the cross-reactivity, sera from 10 patients and 2 tolerant controls were examined individually for specific IgE-binding by IgE ELISA inhibition (Fig 3). The percentage of recognition revealed that Gad c 1 was recognized by sera of 10 patients (100 % recognition). Sal s 1 was the least recognized (60 %). No Sal s 1 inhibition of sera from patients #1 and 12 was seen. The c 1, mackerel, and herring have similar recognition (90% of the sera). Eight sera reacted with wolffish and tuna, whereas halibut and flounder were bound by sera from 7 patients. The recombinant allergens rGad c 1 T1, rSal s 1 14.1, and rThe c 1 P1 were recognized by 7, 8 and 5 sera, respectively. Patient #4 with an anaphylactic reaction showed monospecific sensitivity to Gad c 1 but not to other fish extracts (histogram not shown).

SPT

Further assessment of the cross-allergenicity by SPT was performed by using Gad c 1, Sal s 1 and The c 1 and recombinant allergens (rGad c 1 T1, rSal s 1 14.1, rSal s 1 24.1, and rThe c 1 P1). Nine out of 10 patients displayed positive SPT reactions against the native allergens (Table II), whereas 1 patient showed response to rGad c 1 T1, 8 to rSal s 1 14.1, 1 to rSal s 1 24.1, and 1 to rThe c 1 P1. Remarkably, only 1 patient (#7) reacted to all native as well as recombinant parvalbumins. SPTs on the forearms of this patient and another patient (#9) are illustrated in Fig 4. Seven patients (#1, 2, 3, 5, 7, 11, and 12) were also tested by using herring, wolffish, tuna, and mackerel

TABLE II. SPT results*

	SPT: mean values (mm) of perpendicular diameters of the wheal											
Patient no.	**nGad c 1**	**nSal s 1**	**nThe c 1**	**rGad c 1**	**rSal s 1 14.1**	**rSal s 1 24.1**	**rThe c 1**	**Herring**	**Wolffish**	**Mackerel**	**Tuna**	**Positive control (histamine)**
1	13.25	18.50	11.50	0	4.25	0	—	12.25	14.00	10.75	6.00	5.25
2	11.25	9.00	6.50	0	5.00	0	—	9.25	9.75	3.75	2.50	4.75
3	8.75	12.50	9.75	0	3.50	0	—	9.25	5.50	1.75	1.25	4.50
4	0	0	0	0	0	0	—	—	—	—	—	5.00
5	12.25	9.50	9.25	0	4.75	0	—	5.25	7.75	2.00	0	6.00
7	8.50	8.00	7.50	5.25	5.00	4.75	4.75	10.00	11.50	5.50	4.50	5.75
8	9.00	6.25	6.00	0	4.25	0	0	—	—	—	—	3.25
9	9.25	10.75	8.25	2.75	3.50	0	0	—	—	—	—	5.00
11	5.25	5.50	8.50	0	3.00	1.25	0	9.25	8.50	5.50	2.25	4.50
12	7.75	7.75	8.00	0	0	0	0	10.00	6.25	4.50	2.25	4.75

*Wheal-and-flare responses were measured after 15 minutes. *N*, native; —, not performed.

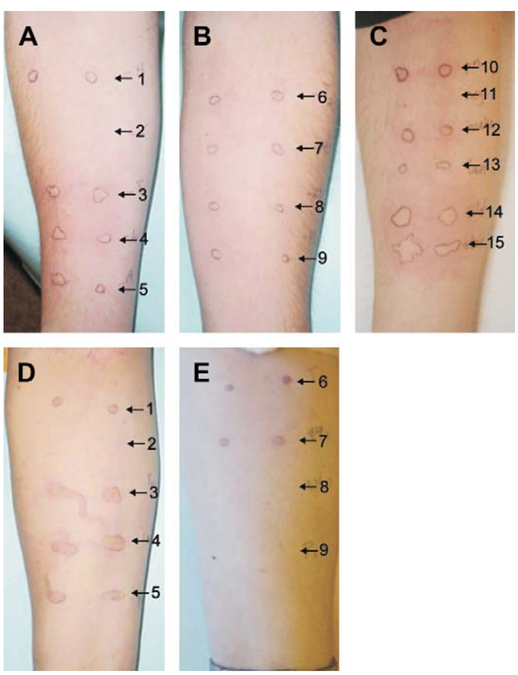

FIG 4. SPTs were performed on the forearms of patients #7 **(A-C)** and #9 **(D and E)**. *1,* Positive control; *2,* negative control; *3,* Gad c 1; *4,* Sal s 1; *5,* The c 1; *6,* rGad c 1 T1; *7,* rSal s 1 14.1; *8,* rSal s 1 24.1; and *9,* rThe c 1 P1. Patient #7 showed similarly high skin responses for mackerel *(12)*, tuna *(13)*, herring *(14)*, and wolffish *(15)*.

extracts. Herring and wolffish could provoke strong reactions in all tested patients. Six patients showed weak or negative responses to tuna and mackerel. The SPT results showed good agreement with the laboratory analysis of serum-specific IgE (Table I) and the ELISA inhibition assays (Fig 3). The serum-specific IgE values of the patients with positive SPT varied from 0.50 to >100 kU/L for Gad c 1 and Sal s 1. One patient who demonstrated negative response in SPT (#4) also had very low serum-specific IgE value for Gad c 1 and Sal s 1. The same patient showed similar finding in IgE ELISA inhibition assay. None of the 10 control individuals showed any wheal or adverse reactions after SPT with recombinant parvalbumins (rGad c 1 T1, rSal s 1 14.1, rSal s 1 24.1, and rThe c 1 P1).

DISCUSSION

Patients examined with cod allergy were particularly sensitized to cod, salmon, and other fish species (Table I). Gad c 1 followed by The c 1 parvalbumins are the ones that showed the highest IgE-binding affinities. The least allergenic fish in this population were halibut, flounder, tuna, and mackerel. This suggests that some of the investigated parvalbumins are highly homologous and share several identical IgE-binding epitopes.[14-16,27] Faint stained proteins other than parvalbumins were seen in the IgE-immunoblots of the allergen extracts (Fig 1, *B*). The allergenicity of the higher molecular weight (>40 kd) IgE-binding proteins has been reported.[28]

The c 1 was found to be the fish allergen that possesses a high capability of inhibiting IgE-reactivity to Gad c 1 in patients' sera; this was similarly reported in a previous study.[25] IgE-binding patterns are more similar when fish species have closer phylogenetic relation and parvalbumins with high amino acid sequence homology.[8,11]

It was shown in a previous study that salmon (Sal s 1) was a less potent allergen than cod and that it could be tolerated, because less than 50% of the patients with cod allergy studied (11 out of the 24 children with cod allergy) reacted to salmon.[18] In the current study, 9 of 10 allergic to cod had serum-specific IgE to salmon. This increase in salmon sensitization during the last 4 decades can probably be explained by its ascending consumption as a result of modern salmon farming, which led to a decline in its market price. Statistical data showed that in 2000 to 2003, the Norwegian salmon consumption increased by 36%.[29] Studies with a larger number of patients seem necessary to allow evidence of the increased prevalence of IgE-mediated salmon allergy.

In a recent article, parvalbumin was also demonstrated to be the major allergen in mackerel.[30] In the current study, mackerel displayed a low degree of cross-reactivity to other fish species. SDS-PAGE of mackerel allergen revealed a weak band at parvalbumin's chromatographic location but negative IgE-binding in immunoblots using patient IgE serum pool (Fig 1, *A and B*).

Herring has been reported to be the cause of occupational cell-mediated contact allergic dermatitis with cross-reactivity to other fish belonging to the Clupeiformes order such as sardine and anchovy.[31] A similar observation was found in this study, suggesting homologous IgE epitopes between herring and cod parvalbumins.

Tuna was one of the least potent in ELISA inhibition. Oral provocations could permit safe ingestion of tuna for some patients with cod allergy. This test was not systematically performed on the studied population because of their inherent risk of anaphylactic reaction. Tuna showed no visible bands in the parvalbumin region (Fig 1). An allergen of about 46 kd was detected in yellowfin tuna[32]; this did not belong to the parvalbumin group, explaining tuna's weak allergenic capacity.

The allergenicity of the recombinant allergens was also determined by IgE ELISA inhibition and SPT. Recombinant Gad c 1 T1, rSal s 1 14.1, and rThe c 1 P1 were recognized by 7, 8, and 5 sera, respectively, in ELISA inhibition. The numbers of positive SPTs with the recombinant allergens were 8 patients for rSal s 1 14.1 and 1 patient for rGad c 1 T1, rSal s 1 24.1, and rThe c 1 P1. This was probably a result of considerably high amino acid sequence variability (32% for Sal s 1 and 38% for The c 1).

IgE cross-reactivity with Gad c 1, Sal s 1, and The c 1 was shown by serological analysis and SPT (Tables I and II). The amino acid sequence of Gad c 1 showed high identity indices with those of parvalbumins from Sal s 1 (68%) and The c 1 (62%), supporting the cross-reactivity between these fish. The poor responses obtained by the recombinant parvalbumins could be caused by conformational masking of high-affinity IgE-binding motifs.

Other studies have examined fish allergy and cross-reactivity in adults and children. Oral challenges on adults showed that Gad c 1 was a reliable marker for fish allergy, but patients could tolerate other species with no adverse reactions. Using DBPCFC, similar conclusions were derived, advising patients to avoid cod and other fish until they got experience that one or several species could be eaten.[15,16,19]

In conclusion, cod, salmon, pollack, herring, and wolffish contained the most potent cross-reacting fish parvalbumins, whereas halibut, flounder, tuna, and mackerel were the least allergenic in the current study. Mackerel and tuna could be tolerated by 7 and 2 patients, respectively, as suggested by very low specific IgE and negative SPT. No DBPCFC was performed on this population because of the high risk of anaphylaxis. Pollack parvalbumin showed high inhibition of cod specific IgE. IgE-binding patterns of fish parvalbumins were strong whenever close phylogenetically relationships existed. Recombinant cod and salmon were allergenically potent, as demonstrated by at least 7 of the 10 sera examined (Fig 3). Their relatively weak allergenicity was suggested to be a result of masking of essential conformations of some high-affinity IgE-binding motifs. Recombinant parvalbumins tested showed no wheal or adverse reactions in SPT of 10 control individuals. Cross-reacting IgE-binding epitopes of recombinant Gad c 1, Sal s 1, and The c 1 could be useful tools for understanding the physiological function of IgE. Two peptides of an intestinal helminth parasite (Sj 22-6) were homologous to IgE-binding epitopes of Gad c 1 and β-lactoglobulin,[33] suggesting that the induction of IgE-mediated allergy and protective immunity are structurally linked. Other potential benefits of cloning and expressing fish recombinant parvalbumins are (1) development of genetically modified hypoallergenic fish, as in the case of peanut, soy, and shrimp[34]; (2) avoidance of incorporating fish homologous epitopes in genetically modified crops[34,35]; and (3) establishment of reliable analytical methods for diagnosis and treatment of fish hypersensitivity.

We thank Judit Eriksen, Ann Kristin Gulliksen, and Ines Sizifredo Gouveia for their technical assistance, and Agnete Hvidsten and Sigrid Løken for performing the SPT. We also thank all patients and healthy volunteers who participated in this study.

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