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Comparison of pediatric and adult IgE antibody binding to fish proteins

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Background: Allergic reactions to fish are a common cause of food allergy.

Objective: We compared the binding of pediatric and adult fish-allergic patient IgE antibodies to fish proteins.

Methods: Clinical histories of fish allergy were confirmed by prick skin tests, RAST and if possible, with blinded oral food challenges. The patients included five children with severe allergic reactions to catfish (4/5), cod (1/5), and tuna (1/5) and five adults with severe allergic reactions to catfish (5/5), cod (2/5), snapper (3/5), and tuna (2/5). Extracted proteins from catfish, cod, snapper, and tuna were separated with SDS-PAGE. IgE immunoblots and immunoblot inhibition studies were performed using serum sample from these patients.

Results: Multiple fish proteins ranging from 12 to 45 kD from the four fish extracts were identified by SDS-PAGE. A major protein (12.5 kD) was present in all fish extracts except for raw tuna. Immunoblots using individual pediatric and adult serum samples revealed that the major IgE binding was to the 12.5-kD protein from catfish, cod, and snapper. The immunoblot with tuna using serum from a pediatric patient with isolated tuna anaphylaxis revealed an IgE binding protein band at 40 kD. Preincubation of serum samples from two separate fish-allergic patients with 1 mg of cod fish extract completely inhibited IgE binding to the 12.5-kD fish protein in subsequent immunoblots.

Conclusions: Pediatric and adult fish-allergic patients have similar in vitro IgE binding to a 12.5-kD protein from fish extracts. This protein is immunochemically similar to Gad c I, the major allergen in cod.

Ann Allergy Asthma Immunol 1997;79:131–7.

INTRODUCTION

Allergic reactions to fish are a common cause of food allergy in pediatric and adult populations.[1,2] These food reactions usually follow the ingestion of fish but have also been reported following the inhalation of vapors generated during the cooking of fish.[3–5] A fatality has even been reported in a fish-allergic patient following the ingestion of a non-fish containing meal that was cooked or fried in oil previously used to fry fish.[6]

Fish allergy is more prevalent in European countries where the consumption and processing of fish are more common.[1,7–10] The ingestion of fish in the United States is increasing, most likely because of health concerns.[11] Americans consumed 10.3 pounds of fish and other seafood per person per year in 1960, whereas statistics from 1992 reveal a consumption rate of 14.7 pounds of fish and other seafood per person (US Dept Commerce, personal communication). This represents an almost 50% increase in fish consumption over the last 30 years. Despite this trend, fish allergy in the United States has not been widely studied.

A significant percentage of children with allergies to egg, cow milk, soybean, and wheat lose their clinical reactivity by late childhood.[12,13] In contrast, the natural history of fish hypersensitivity parallels that of peanut and tree nut allergies, which are typically lifelong sensitivities.[14–16] One exception to this was an investigation by Kajosaari that reported the symptoms of fish allergy in children may diminish with age.[17] While studies of fish hypersensitivity have been performed using either pediatric or adult patients,[1,18] there is no comparison of the specific immunologic features of fish-induced allergic reactions between pediatric and adult patients. The purpose of this study was to compare the serum IgE antibody binding to fish proteins between pediatric and adult fish-allergic patients.

MATERIALS AND METHODS

Patient Recruitment and Normal Controls

Pediatric and adult patients with histories of fish allergy were recruited from the allergy clinics at the University of Arkansas for Medical Sciences/Arkansas Children's Hospital and Tulane University Hospital, respectively. Two fish-tolerant subjects, an atopic pediatric patient with hyper-IgE syndrome and an atopic adult with a high serum IgE concentration, were used as controls. Serum samples were collected on all subjects and stored at −20 °C until used in specific immunologic studies. Informed consent was obtained for each subject or from their parents. All studies were approved by the Human Research Advisory Committee at the respective institutions.

Preparation of Fish Extracts

Commercial extracts of raw catfish (1:20 wt/vol Greer Laboratories, Lenoir, NC), cod (1:10 wt/vol Center Laboratories, Port Washington, NY), and snapper (1:10 wt/vol Hollister-

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The authors are supported in part by grants from the Allergy and Immunology Institute of the International Life Sciences Institute (JMJ), from the Allergy and Asthma Foundation of America (AWB), and from the National Fisheries Institute (SBL).

Received for publication November 4, 1996.

Accepted for publication in revised form February 26, 1997.

Stier, Spokane, WA) were dialyzed against PBS, concentrated by dialysis with Ficoll 400 (Pharmacia LKB, Uppsala, Sweden) and stored at −20 °C. These commercial fish extracts were used for skin testing all of the children and adults with fish allergy. Extracts of raw tuna fillet, purchased from a local seafood store (New Orleans, LA), were prepared by homogenization of 500 g in 1 L 0.1 M phosphate-buffered saline (PBS) (pH 7.2) in a Waring blender (New Hartford, CT) for one to three minutes at room temperature. Homogenates were extracted overnight at 4 °C under constant stirring, centrifuged (70,000 *g*) and the supernatants concentrated on an Amicon-YM2 filter (Amicon, Danvers, MA; M.W.C.O. <1 kD). The concentrates were re-centrifuged (180,000 *g*) and the supernatants were aliquoted and stored at −20 °C. Protein concentrations were measured by a phenol reagent method (Sigma Diagnostics, St. Louis, MO). All of the serologic and immunoblot investigations were performed using the PBS-extracts of raw tuna fillet.

Skin Testing with Fish Extracts

All patients, including some with histories of severe allergic reactions including anaphylaxis in some cases following fish ingestion, were required to discontinue antihistamine therapy for 7 to 14 days before skin tests were performed by the prick or puncture method. Standardized, glycerinated fish extracts were used as follows at Arkansas Children's Hospital 1:20 (wt/vol) Greer Laboratories, and at Tulane University: catfish 1:20 (wt/vol), Greer Laboratories, cod 1:10 (wt/vol), Center Laboratories and snapper 1:10 (wt/vol) Hollister-Stier, and tuna 1:10 (wt/vol) Center Laboratories. Individual extracts were applied to the patient's back or volar surface of the forearm by a clinical research nurse as previously described.[19,20] Skin test results were considered positive when a food antigen elicited a wheal of at least 3 mm greater than the control prick (50% glycerinated saline).

Radioallergosorbent Testing (RAST) with Fish Extracts

All of the RASTs with fish extracts were performed at Tulane University using individual serum samples from the pediatric and adult study patients. In brief, cyanogen bromide-activated paper disks,[21] coated with fish extract (50 μg protein/disk), were incubated overnight in duplicate with 100 μL undiluted patient serum. After washing with saline (0.9%), 100 μL of I^{125}-labeled anti-IgE (15,000 cpm/disk; Kallestad, Chaska, MN) were added and incubated overnight. Disks were washed and bound ^{125}I (cpm) measured in a gamma counter (Gamma 5500, Beckman, Irvine, CA). Results were expressed as mean percent binding of total radioactivity added and RAST% binding ≥ 3% was considered positive. Based on previous experience with this system,[22,23] binding ≥ 3% represents three standard deviations from mean percent binding obtained with serum samples of atopic control subjects who were skin test negative to the allergens under investigation.

Blinded Challenges to Fish in Pediatric Patients (University of Arkansas for Medical Sciences/Arkansas Children's Hospital)

The double-blind placebo-controlled food challenges (DBPCFC) were performed in pediatric subjects as described previously.[13,18,24] Positive reactions were established when cutaneous, nasal, pulmonary, or gastrointestinal tract symptoms were observed within two hours of the DBPCFC. Any significant delayed symptoms within 24 hours of the food challenge were also reported. All negative DBPCFC were confirmed by an open feeding of approximately 6 oz of the baked fish under investigation. Pediatric patients with convincing histories of severe anaphylaxis (wheezing, laryngospasm, and hypotension) following specific fish ingestions were not subjected to DBPCFC. After a diagnosis of fish allergy was confirmed, all study patients were placed on an appropriate fish-elimination diet.

Blinded Challenges to Fish in Adult Patients (Tulane University Hospital)

The challenge protocol for adults consisted of randomly administering increasing amounts of a specific fish (1, 4, 16, and 64 g) disguised in ground turkey and A-1 steak sauce or placebo samples (only ground turkey and A-1 steak sauce) with an hour interval between each ingestion.[3] As in the pediatric DBPCFC, all food samples were prepared and randomized by the same person who did not participate in the actual food challenges. The initial challenge dose administered was based on the patient's history of sensitivity, with lower doses of less than 1 g given to those with a prior history of a severe reaction. Vital signs and peak flows were obtained prior to ingestion of each dose and one hour after completion of the challenge. Throughout the challenge, subjective and similar objective signs and symptoms, as described above in the pediatric challenges, were noted for time, duration, severity, and resolution. Patients were also given a questionnaire to report any delayed signs or symptoms occurring within 24 hours after discharge. All negative DBPCFC were confirmed by an open challenge feeding of 64 grams (approximately 2 oz) of the baked, unseasoned fish under study. As above, adult patients with the most severe convincing histories of anaphylaxis (wheezing, laryngospasm, and hypotension) were not subjected to DBPCFC. After a diagnosis of fish allergy was confirmed, all study subjects were placed on an appropriate fish-elimination diet. The ultimate decision to challenge the pediatric and adult patients was based on the clinical history of fish allergy. If there was a convincing history of generalized anaphylaxis, which was life-threatening, after the ingestion of a specific fish species, we did not perform a blinded food challenge. If the clinical history was not convincing for anaphylaxis or not life-threatening, we performed a blinded food challenge to confirm the patient's clinical reactivity to the fish. We did not use positive results of skin tests or

Table 1. Clinical Summary of Patients with Fish Allergy*

Patient	Age, yr	Sex	Clinical History of Fish Allergy	PST Positive	RAST Positive	Challenge Positive
Pediatric patients						
1	2	F	generalized urticaria and angioedema to CF	CF, C	CF, C	CF
2	2	F	exacerbations atopic dermatitis to C, CF	CF, C	CF, C	CF, C
3	8.5	F	anaphylaxis to CF	CF, C, T	CF, C, S	convincing history
4	5	M	anaphylaxis to CF	CF, C	CF, C	convincing history
5	12	M	anaphylaxis to T	T	CF, C, T	convincing history
Adult patients						
6	39	F	anaphylaxis to S	CF, C, S, T	CF, C, S, T	CF, C, S, T
7	36	M	anaphylaxis to CF, S, T	CF, C, S, T	CF, C, S	convincing history
8	25	F	anaphylaxis to CF, C, S	CF, C, S, T	CF, C, S, T	CF, C, S
9	56	F	anaphylaxis to CF	CF, C, T	CF, C, S	convincing history
10	20	F	urticaria to CF	CF, C, S, T	C, S	convincing history

* PST = prick skin test, RAST = radioallergosorbent assay, CF = catfish, C = cod fish, S = snapper, and T = tuna.

RAST in isolation to determine whether or not to perform the food challenges.

SDS-PAGE of Fish Protein Extracts

All of the SDS-PAGE and subsequent IgE immunoblotting investigations were performed at Arkansas Children's Hospital. Fish extracts, preparation described above, were separated by SDS-PAGE using a the method described by Laemmli et al.[25] In brief, the protein samples, 20 μg and molecular weight standards (BioRad, Hercules, CA) were resolved in a 3% stacking gel and a 10% separating gel at 20 mA/gel (25 mM Tris and 192 mM glycine buffer with 1% SDS). The individual protein bands were identified with a Coomassie blue stain (Sigma Chemical Co, St Louis, MO).

IgE (Western) Immunoblots

Separated fish proteins were transferred from SDS-PAGE gels to a nitrocellulose membrane in a 25 mM Tris/192 mM glycine/40% methanol transfer buffer.[26] The procedure was accomplished using a transblot apparatus (BioRad, Hercules, CA) for two hours at 150 mA. After a blocking step (0.5% gelatin in PBS) for 16 hours, the nitrocellulose blot was washed three times each with PBS containing 0.05% Tween 20 (PBST) and then probed with individual patient serum samples (1:20 vol/vol dilution) for two hours at 23 °C while rocking for 16 hours at 4 °C. After three separate washes with PBST, ^{125}I-labeled, equine antihuman IgE antibody (Kallestad, Chaska, MN) was added (1:3 vol/vol PBS) and incubated at 23 °C while rocking for two hours. Following a second wash step, the blot was placed on X-ray film at −70 °C for 12 to 24 hours and then developed in an X-Omatic processor.

Immunoblot Inhibition with Cod

Immunoblot inhibition studies were performed by preincubating fish-allergic patient serum with 1 mg of cod fish extract for four hours at 23 °C. Cod fish extract was selected because a major 12.3-kD allergen, Gad c I, has been identified from this fish species. Lysozyme (MW of 14.4 kD), a protein of similar molecular weight to Gad c I, was used as a negative control.

RESULTS

Five fish-allergic children ranging in age from 2 to 12 years (median age 5 years) were studied at Arkansas Children's Hospital (Table 1). These pediatric patients had histories of allergic reactions following the ingestion of catfish (4/5), cod (1/5), and tuna (1/5). All five patients had positive prick skin

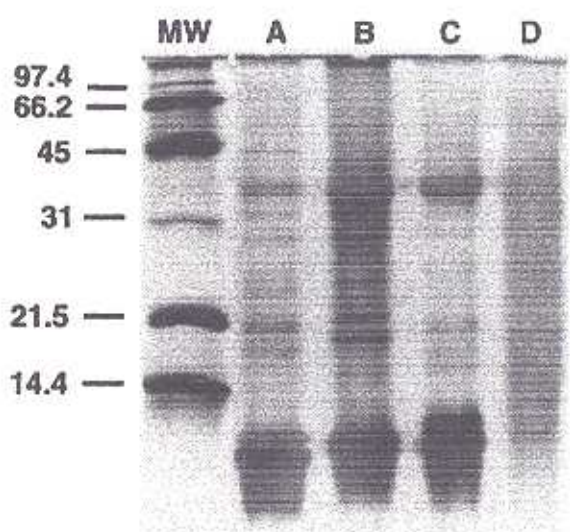

Figure 1. Multiple fish proteins extracted from catfish (A), cod (B), snapper (C), and tuna (D) were separated by SDS-PAGE and stained with a Coomassie blue stain. They are compared with standard molecular weight markers (MW).

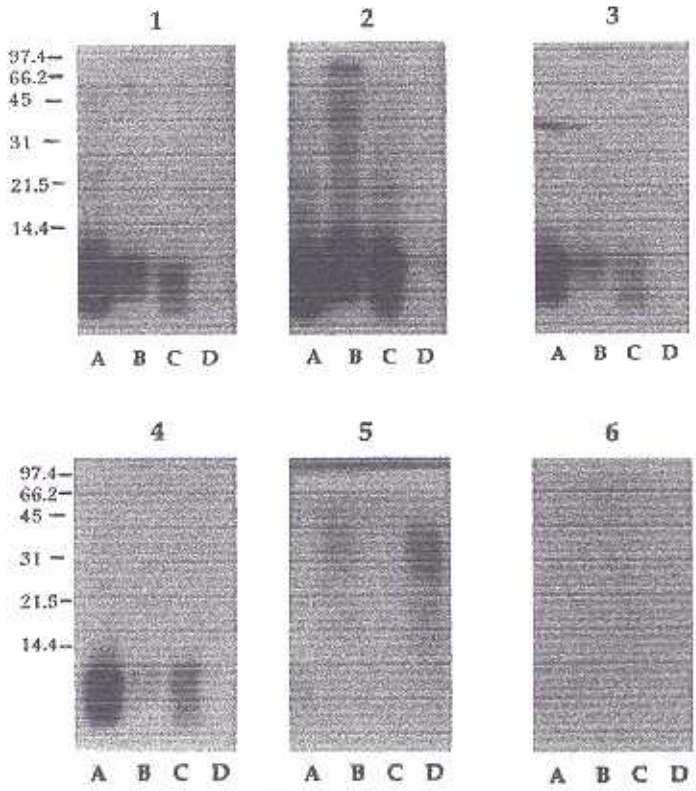

Figure 2. Panels 1–4 represent four individual pediatric patient IgE immunoblots demonstrating the major IgE binding to a 12.5-kD protein present in catfish (A), cod (B), and snapper (C), but no binding to proteins in the raw tuna (D). Representative molecular weights are labeled on the left. Panel 5 represents an IgE immunoblot from a tuna-allergic pediatric patient revealing the major IgE binding at approximately 40 kD. Panel 6 represents an IgE immunoblot using a pediatric control patient that reveals no IgE binding to any of the fish proteins.

tests and/or RAST to fish extracts. Of the five children, three had convincing histories of severe anaphylaxis to fish and two children had their histories of fish allergy confirmed with a positive DBPCFC. In addition, five fish-allergic adults ranging in age from 20 to 56 years (median age: 36 years) were identified at Tulane University Medical Center (Table 1). These adult patients had clinical histories of anaphylaxis following the ingestion of catfish (4/5), cod (1/5), snapper (3/5), and tuna (1/5). All five adult patients had positive prick skin tests and/or RAST to different fish extracts. Positive DBPCFC to the suspect fish species were confirmed in two of these patients, one of whom had a positive challenge to tuna despite no clinical history of tuna allergy. The remaining three adults were not challenged because of significant clinical histories of fish anaphylaxis. There were no significant delayed reactions to any of the blinded fish challenges performed in the study patients. Finally, there were no adverse reactions observed during any of the placebo food challenges or in any open challenges performed subsequently to negative DBPCFC in the pediatric or adult patients with fish allergy.

Multiple fish proteins ranging from 12 to 45 kD were identified by Coomassie blue staining of 10% gels of catfish, cod, snapper, and tuna extracts (Fig 1). A major protein band of approximately 12.5 kD was present in the extracts of catfish, cod, and snapper but was absent in the extract from raw tuna.

Results of individual IgE immunoblots performed using serum samples from the pediatric patients are demonstrated in Figure 2. The major IgE binding from four of the five patient IgE immunoblots was to a 12.5-kD protein present in catfish, cod, and snapper (Fig 2, panels 1–4). These four patients did not show IgE binding to proteins in the raw tuna. Interestingly, the remaining pediatric patient had positive RAST to catfish, cod, and tuna but only a clinical history of anaphylaxis following ingestion of tuna. His IgE immunoblot revealed the major IgE binding at approximately 40 kD (Fig 2, panel 5). Finally, an immunoblot using serum from the pediatric control patient revealed no IgE binding to any of the fish proteins (Fig 2, panel 6).

Results of individual IgE immunoblots using serum samples from five adult patients are summarized in Figure 3. The major IgE binding in all of these patients was to a 12.5-kD protein present in catfish, cod, and snapper (Fig 3, panels 1–5). No binding was demonstrated to any raw tuna proteins. Finally, immunoblot using serum from the adult control patient revealed no IgE binding to any fish proteins (Fig 3, panel 6).

To determine whether the 12.5-kD fish protein responsible for the major IgE binding in these studies was immunochemically similar to Gad c I, immunoblot inhibition studies were performed. Two fish-allergic patient sera, one pediatric and one adult, were preincubated with 1 mg cod fish extract. Subsequent immunoblots revealed complete elimination of IgE binding to 12.5-kD fish proteins equivalent in size to Gad c I. This is demonstrated in the pediatric fish-allergic patient in Figure 4 (panel 2). Patient sera that was preincubated with either lysozyme (Fig 4, panel 3), a 14.4-kD protein of similar molecular weight to Gad c I, or PBS buffer alone (Fig 4, panel 1) did not inhibit IgE binding.

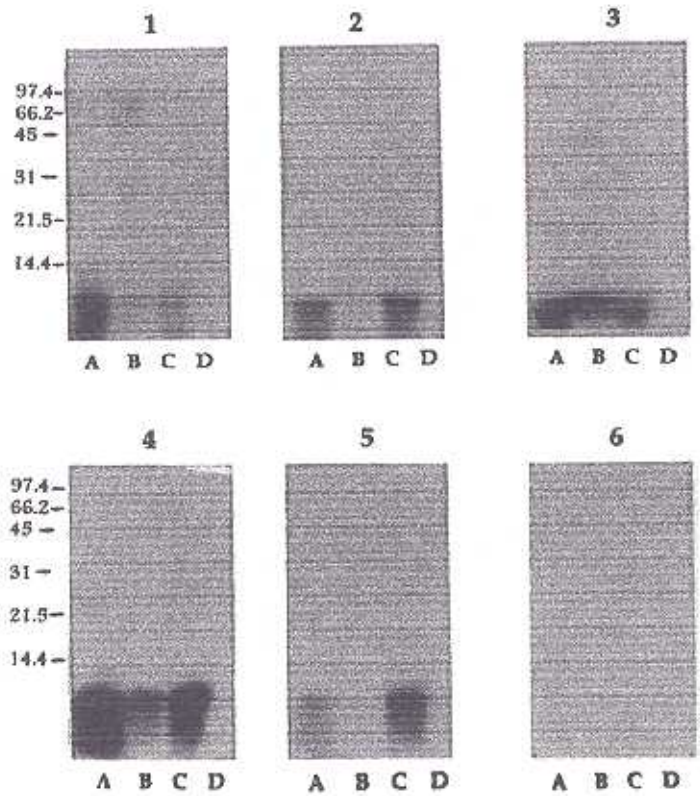

Figure 3. Panels 1–5 represent five individual adult patient IgE immunoblots demonstrating the major IgE binding to a 12.5-kD protein present in catfish (A), cod (B), and snapper (C), but no binding to proteins in raw tuna (D). Representative molecular weights are labeled on the left. Panel 6 represents an IgE immunoblot using an adult control patient that reveals no IgE binding to any of the fish proteins.

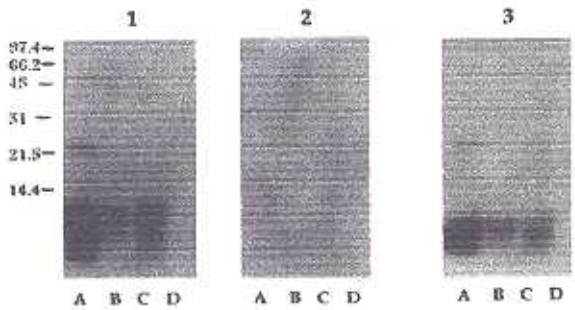

Figure 4. Immunoblot inhibition studies using cod fish extract and serum from one representative pediatric fish-allergic patient reveal complete elimination of IgE binding to 12.5-kD fish proteins equivalent in size to Gad c 1 (panel 2). Patient serum that was preincubated with either lysozyme (panel 3) or PBS buffer alone (panel 1) did not inhibit IgE binding. Representative molecular weights are labeled on the left.

DISCUSSION

The results from this study demonstrate that pediatric and adult fish-allergic patients have similar in vitro IgE antibody binding to fish proteins. The predominant IgE binding was to a 12.5-kD protein in the catfish, cod, and snapper. This protein is similar to Gad c 1, the major 12.3-kD allergen that has been isolated from cod. Extracts from raw tuna lacked this 12.5-kD protein band, but contained a high molecular weight protein band of approximately 40 kD responsible for specific IgE binding in a tuna-allergic pediatric patient.

Previous investigations using serum samples from fish-allergic, pediatric patients have revealed IgE binding to proteins from a variety of fish species.[18,27] These authors demonstrated that a major fish protein, of approximately 13 kD, was responsible for the majority of the specific IgE binding in their pediatric fish-allergic patients. This protein was absent in raw and cooked tuna extracts and albacore, a fish species similar to tuna.[27] A report from Pascual and co-workers[1] and our study confirm these findings. Furthermore, our findings are extended to fish-allergic, adult patients whose specific IgE binding was very similar to that observed in fish-allergic pediatric patients.

Gad c 1, known previously as allergen M,[2,28–31] is a calcium-binding protein with an estimated molecular weight of 12.3 kD and an isoelectric point of 4.75. This food allergen belongs to the parvalbumins found in fish muscle tissue.[3] The primary amino acid structure, consisting of 113 amino acid residues, and short synthetic peptides of this allergen have been shown to bind IgE. Further, Gad c 1 appears to be the immunodominant antigen in cod, and its homologue in other fish species may help explain multiple fish sensitivities commonly observed in clinical practice.[4,32] Results from the immunoblot inhibition experiments in our study suggest that a 12.5-kD fish protein, immunochemically similar to Gad c 1, is the major IgE binding protein from catfish, cod, and snapper in both pediatric and adult fish-allergic patients.

Multiple fish sensitivity as determined by in vitro laboratory tests is not uncommon, but caution must be used in the clinical interpretation of these tests. Pediatric patients allergic to one fish can often eat other fish species

without experiencing allergic reactions.[4,7,18] In contrast, recent adult studies suggest that there is significant clinical cross-reactivity among different fish species.[33,34] In the current study, pediatric and adult patients had positive prick skin tests and specific IgE binding to fish proteins from multiple fish species. We cannot comment on the specificity of fish allergy, however, because DBPCFC were not performed on all the patients with the individual fish species. Most importantly, the ultimate clinical decision regarding restriction of fish from the diet should be based on the results of oral food challenges.

As reported previously,[22] canned tuna extracts lack definable protein bands, especially those less than 20 kD, that may be responsible for IgE-mediated allergic reactions. Commercial processing including the use of high temperature in preparing canned tuna products may provide an explanation for this lack of definable protein bands. Canned tuna might, therefore, be a low-fat, nutritious protein food alternative for some fish-allergic patients. In our study, extracts of raw tuna lacked any identifiable protein band at 12.5 kD but did contain several protein bands of higher molecular weights. This may be a result of our using raw tuna, instead of cooked tuna, for the actual extraction material.

Prospective studies using standardized food challenges are needed to characterize the clinical course of pediatric fish-allergic patients as they approach their adult years and would be helpful in defining the species-specificity of fish allergy. In addition, future investigations should be directed at producing recombinant fish allergens, for example Gad c 1 or its homologue in other fish species, to help characterize and map the important allergenic determinants from different fish species. Direct applications from this work should provide more specific diagnostic tests for fish hypersensitivity and would help identify patients truly at risk for these reactions. In addition, identifying fish species with less allergenicity and exploring fish processing measures that reduce allergenicity would be logical research applications. With a better understanding of the allergic response to fish allergens, potential therapeutic options for fish-allergic patients, typically a life-long sensitivity with a potential for serious life-threatening reactions, may become available. Because both pediatric and adult fish-allergic patients recognize one major fish allergen, this disorder should provide a useful model to investigate potential diagnostic tests and therapeutic options.

ACKNOWLEDGMENTS

We are indebted to Marjorie McCants and Jason Guin for their technical assistance in performing these studies.

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