Fish hypersensitivity. II: Clinical relevance of altered fish allergenicity caused by various preparation methods

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In double-blind, placebo-controlled, oral food challenges with fish, a 12-fold higher false-negative rate was found compared with other food antigens. In an effort to elucidate this discrepancy, cooked lyophilized fish extracts (used in double-blind, placebo-controlled, oral food challenges) were compared with cooked, nonlyophilized fish extracts (used in open challenges) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblot, and ELISA-inhibition assays. Altered fish allergenicity as a result of food processing was examined with canned tuna and salmon. Forty-five children and young adults with food allergies, including 18 patients with IgE-mediated hypersensitivity to fish, were challenged with canned tuna. All 45 challenges with canned tuna were negative. Two of these patients are allergic to salmon and also have negative reactions to challenges with canned salmon. In vitro investigation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of tuna and salmon extracts revealed a striking loss of definable protein fractions in the canned fish extract when compared with raw and cooked fish extracts, and immunoblot analyses demonstrated minimal IgE-specific binding to the canned fish extracts. In addition, decreased allergenicity of the canned tuna and salmon was demonstrated by ELISA-inhibition assay and by negative oral challenges with canned salmon in two patients allergic to salmon. Collectively, these findings suggest that some of the major allergens responsible for IgE-mediated food allergy to fish are more labile than previously recognized. (J ALLERGY CLIN IMMUNOL 1992;90:622-9.)

Key words: Fish allergy, food hypersensitivity, double-blind, placebo-controlled oral food challenges, lyophilization, ELISA—inhibition assay, immunoblotting

Allergenic food proteins are characteristically heat and acid stable, largely resistant to proteolysis and digestion, and their primary amino acid structures are bound by IgE. Indeed, these inherent physicochemical characteristics of allergenic food proteins may play a major role in their allergenicity. Furthermore, despite the wide variety of foods consumed, only a small number are responsible for more than 90% of the food-allergic reactions in both pediatric and adult populations. Unfortunately for the population with food allergies, most allergenic food proteins require extensive alteration to decrease their allergenicity. This is an issue that has gained notoriety in the infant formula...
market. Fish antigens are thought to be no exception; Gad C1 from codfish, the best characterized food allergen, exemplifies the qualities of a classic food allergen as it resists digestion, cooking, and proteolysis, and its primary structure is allergenic. Given the stability of Gad C1, the finding that 7 of 33 (21%) false-negative, double-blind, placebo-controlled, oral challenges (DBPCFCs) with cooked, lyophilized (freeze dried) fish was especially unexpected.

The characteristics of the food allergens responsible for the clinical entity “oral allergy syndrome” appear quite different. Oral allergy syndrome is seen most often with the ingestion of raw apples, carrots, potatoes, and hazel nuts in patients with inhalant allergy to birch, and melons and bananas in patients with inhalant allergy to ragweed. It is thought to be caused by an IgE-mediated response to food allergens that are extremely labile and can be destroyed with minimal processing.

This study describes physicochemical and immunologic evidence for decreased allergenicity and stability of fish proteins resulting from canning and lyophilization and the clinical implications of these changes.

MATERIAL AND METHODS

Effects of lyophilization on fish allergens. Extracts were made to investigate the false-negative DBPCFCs to flounder, perch, cod, and salmon that resulted from our initial fish hypersensitivity study. To mimic the DBPCFCs the fish were cooked and lyophilized before extraction of proteins, and to mimic the open challenges the fish were cooked and the proteins extracted without lyophilization. The extracts were prepared by adding 10 gm of fish separately to 40 ml of phosphate-buffered saline in sterile centrifuge tubes with a mixing glass marble. The tubes were placed on a rotator in a cold room (4°C) overnight. Fat, if present, was skimmed off after initial extraction and after each centrifugation. The extracts were centrifuged at 1250 g for 15 minutes and then clarified at 16,000 g for 15 minutes as previously described.

Physicochemical properties of the cooked, nonlyophilized versus the cooked, lyophilized extracts from flounder, perch, cod, and salmon were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as previously described. In brief the fish extracts were reduced in SDS sample buffer and boiled for 10 minutes, and then they were resolved in a discontinuous SDS-PAGE system. The blocked fish nitrocellulose blots were probed for IgE-specific binding with a 1:25 dilution of sera from four patients who had a false-negative fish DBPCFC. Immunochromic analyses of the cooked, nonlyophilized versus cooked, lyophilized fish protein extracts were performed with an ELISA inhibition assay with use of sera from the same four patients who had experienced a false-negative fish DBPCFC.

**Abbreviations used**

- **DBPCFC**: Double-blind, placebo-controlled, oral food challenge
- **SDS-PAGE**: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
- **SPT**: Skin prick test

**TABLE I. Atopic profile of the 45 study patients**

| History of asthma | 30 |
| History of atopic dermatitis | 38 |
| History of allergic rhinitis | 36 |
| Positive family history for atopy | 44 |
| Patients with confirmed food allergy by DBPCFC (Patients previously reactive: 1 egg, 1 milk) | 43 |
| Patients allergic to fish | 18 |
| Documented by oral challenge | 11 |
| Diagnosed by recent convincing histories of fish induced anaphylaxis | 7 |

**Challenge studies with canned tuna.** Forty-five atopic pediatric and young adult patients were studied. All forty-five patients were highly atopic, as outlined in Table I. The patients ranged in age from 15 months to 30 years (median, 6.0 years). All 45 patients were admitted to the Johns Hopkins Pediatric Clinical Research Unit (RRO00532) for oral food challenges. Skin prick tests (SPTs) with a raw tuna (yellow-fin) extract (1:20 wt/vol; Greer Laboratories, Lenoir, N.C.), histamine control (1 mg/ml), and saline control were applied to the forearms or backs of the patients by the prick method on the day of admission. Patients were given 90 to 180 gm of canned tuna: Empress Chunk Light Tuna, salt, water (distributed by Mitsui Foods, Hackensack, N.J.).

**Challenge studies with canned salmon.** Two patients with IgE-mediated allergic reactions (vomiting, oral pruritus, urticaria, and angioedema) to broiled or cooked salmon had SPTs with a raw salmon extract (1:20 wt/vol; Greer Laboratories) and were fed 90 to 180 gm of canned pink salmon (Starkist, Long Beach, Calif.). Symptoms to all challenges were recorded on a standardized symptom score sheet. All challenges took place under observation on the Research Unit. All procedures were approved by the Johns Hopkins University School of Medicine Joint Council on Clinical Investigation.

**Control subjects.** SPTs to raw tuna extract, raw salmon
FIG. 1. Amido black stained immunoblots after separation by 15% SDS-PAGE. Lanes 1 and 2: C, Cooked, nonlyophilized; C, 1, cooked, lyophilized; F, flounder; lanes 3 and 4: P, perch; lanes 5 and 6: CO, cod; lanes 7 and 8: SA, and salmon. 25 μg of protein loaded per gel lane.

extract, histamine control, and saline control were applied to three groups of control subjects: 10 atopic patients with food allergy but not fish allergy, 10 atopic patients with no food allergy, and 10 nonatopic volunteers.

Effects of processing on IgE-binding. The canned tuna and canned salmon extracts were prepared as previously described in the "Materials and Methods" section (effects of lyophilization on fish allergens). Extracts from one lot of 10 different water packs of canned tuna: Giant Chunk Light and Albacore (Giant Food Inc., Washington, D.C.), Starkist Chunk Light, Light PC and Albacore (Starkist), Bumblebee Albacore and Chunk Light (Bumblebee Seafood Inc., San Diego, Calif.), Chicken of the Sea Chunk Light and Albacore (Van Camp Seafood Company Inc., San Diego, Calif.), and Empress Chunk Light, plus two canned salmon: Starkist Pink and Red Salmon, were made. Physicochemical properties of tuna extracts: raw, cooked, and 10 canned packs, and salmon extracts: raw, cooked, canned pink, and canned red were examined by SDS-PAGE and immunoblotting as previously described. The tuna nitrocellulose blots were probed with a 1:25 dilution of sera from seven representative patients who had a positive SPT to raw tuna extract but a negative reaction to open canned tuna challenge. The blocked salmon blots were probed with a 1:25 dilution of sera from three patients allergic to salmon. Immunochemical analyses of the cooked and canned tuna and cooked and canned salmon proteins were performed with an ELISA inhibition assay with use of a previously described method. In brief, either cooked tuna or cooked salmon protein extracts were used to coat microtiter plates. With use of a similar experimental design the inhibition assays were performed by pipetting a fixed-dilution (1:10 or 1:5) of patients' sera into several parallel series of Eppendorf (Netheler Heinz GmbH, Hamburg, Germany) tubes containing 10-fold serial dilutions of either cooked or canned tuna from 1 mg/ml to 1 μg/ml and in the salmon experiment either cooked or canned salmon from 500 μg/ml to 5 μg/ml. The antigen-antibody mixture was then incubated, and 100 μl of this antigen-antibody solution was added to the microtiter wells previously coated with cooked tuna or cooked salmon. The plates were then incubated with absorbed affinity-purified biotinylated goat anti-human IgE (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.), developed, and read. Sera from two patients who had a positive SPT to raw tuna extract, a strongly positive ELISA to cooked tuna, but a negative reaction to open canned tuna challenge, and sera from three patients allergic to salmon were all performed individually with the ELISA-inhibition assay.

RESULTS

Effects of lyophilization on fish allergens. SDS-PAGE and immunoblotting were performed with fish extracts equivalent to those used in the positive open fish challenges (i.e., cooked and nonlyophilized) and compared with fish extracts used in false-negative DBPCFCs (i.e., cooked and lyophilized). No demonstrable differences were noted on SDS-PAGE between the cooked nonlyophilized and the cooked lyophilized extracts from perch, codfish, and salmon (Fig. 1). Examining the six fish extracts on a gel (cooked non-
lyophilized and cooked lyophilized perch, cod, and salmon) at concentrations from 2.5 μg/lane to 40 μg/lane also failed to reveal any notable differences between nonlyophilized and lyophilized fish extracts. Minor differences were apparent between the cooked nonlyophilized and cooked lyophilized flounder extracts. Bands at 23,000 d and 15,000 d were prominent in the nonlyophilized lane but were absent in the lyophilized lane. Three of the seven false-negative DBPCFCs were with flounder. In addition, nitrocellulose blots probed with sera from four patients who had a false-negative fish DBPCFC did not demonstrate any differential binding patterns between the fish extract responsible for the false-negative DBPCFC (cooked and lyophilized) and the fish extract corresponding to the positive oral challenge (cooked nonlyophilized). These four patients all had systemic allergic symptoms with their positive open fish challenge, although two of the reactions began with oral symptoms consistent with the oral allergy syndrome.

Representative immunoblots from two of these patients are shown in Fig. 2. IgE-specific ELISA inhibition assays with sera from these four patients who had a false-negative fish DBPCFC failed to demonstrate any differences in inhibition potency between the cooked, nonlyophilized (open challenge equivalent) versus the cooked, lyophilized (DBPCFC equivalent) fish extracts.

**Challenge studies with canned tuna.** SPTs to the raw tuna extract were positive in 21 of 43 patients (median wheal, 4 mm; range, 3 to 11 mm). SPTs to the raw tuna extract were negative in all 30 subjects in the three control groups. All 45 patients consumed 90 to 180 gm of canned tuna without symptoms. These patients have continued to eat canned tuna for 6 months to more than 2½ years without any history of sensitization or adverse reaction.

**Canned salmon results.** SPTs to the raw salmon extract were positive (mean wheal diameter, 7 mm, 6 mm) in the two patients allergic to salmon who were challenged openly with canned salmon. SPTs to the raw salmon extract were negative in all 30 subjects in the three control groups. These two patients allergic to salmon consumed canned pink salmon without adverse reaction and have been consuming canned salmon for 1 to 2 years without incident.

**Effects of processing on IgE binding.** Fig. 3, A depicts an Amido black protein stain of three tuna extracts (raw, cooked, and canned) run on SDS-PAGE and electrophoretically transferred to nitrocellulose paper. The raw extract (lane 1) has distinct protein bands, the cooked extract (lane 2), in addition to distinct protein bands, has proteins that appear to form high-molecular-weight conglomerates, and the canned extract (lane 3) reveals a striking loss of definable protein bands. The canned tuna extract appears as a near homogenous mixture of different molecular weight fragments. All 10 canned tuna extracts appeared similar. Immunoblots of the raw, cooked, and canned tuna extracts were probed with sera from seven of the 45 patients. These seven patients were allergic to fish, had a positive SPT to raw tuna extract, and had negative open canned tuna challenges. All tuna blots probed with sera from these patients allergic to fish had minimal-to-absent IgE-specific binding to the canned tuna extract. Fig. 3, B, C, and D illustrates representative tuna immunoblots from three of these patients. As shown in Fig. 4, A, SDS-PAGE and immunoblotting of the raw, cooked, and canned salmon (pink and red) extracts showed similar results to those of the tuna extracts, with a marked loss of definable protein bands in the canned pink (lane 3) and canned red (lane 4) salmon extracts. In addition, there was minimal IgE-binding to the canned salmon extracts.
FIG. 3. A, Amido black stained immunoblots of tuna extracts after separation by 15% SDS-PAGE: mw, Molecular weight markers; raw (lane 1), cooked (lane 2), and canned (lane 3). 25 μg of protein loaded per gel lane. B, C, D, Three representative tuna immunoblots developed for IgE-specific binding with sera from three patients with fish allergy who had positive SPTs for tuna and consumed canned tuna without symptoms: raw (lane 1), cooked (lane 2), and canned (lane 3).

FIG. 4. A, Amido black stained immunoblots of salmon extracts after separation by 15% SDS-PAGE: hmw, High molecular weight markers; raw (lane 1), cooked (lane 2), canned pink (lane 3), and canned red (lane 4). 25 μg of protein loaded per gel lane. B, C, Salmon immunoblots developed for IgE-specific binding with sera from two patients with salmon allergy who ate canned salmon without symptoms: raw (lane 1), cooked (lane 2), canned pink (lane 3), and canned red (lane 4).
IgE-specific inhibition ELISAs were performed to further investigate the decreased allergenicity of canned tuna and canned salmon compared with cooked tuna and cooked salmon, respectively. The tuna inhibition ELISAs were performed individually with sera from two study patients with significant amounts of anti-cooked-tuna antibody. Fig. 5 depicts the results of the average inhibition of the tuna assays. Increasing amounts of cooked or canned tuna protein were used to inhibit binding of sera from two study patients with significant amount of anti-cooked-tuna antibody to a microtiter plate coated with cooked tuna. The amount of canned tuna required to achieve 50% inhibition of IgE-specific binding was approximately 200 times the amount required with cooked tuna. Fig. 6 depicts the results of individual inhibition assays in three patients allergic to salmon. Again, increasing amounts of cooked or canned salmon protein were used to inhibit binding of sera from patients with a salmon allergy to a microtiter plate coated with cooked salmon. The amount of canned salmon required to achieve 50% inhibition of IgE-specific binding was 100 to 200 times more than the amount required with cooked salmon.

**DISCUSSION**

Extensive characterization of the major codfish antigen, Gad C1, has been completed. The primary amino acid structure and short synthetic peptides from the major allergenic epitope of Gad C1 bind IgE from sera of patients allergic to codfish. These patients react to the ingestion of as little as 1 mg of purified Gad C1. Given the stability of the major allergen responsible for codfish allergy, and the fact that lyophilized food antigens have been the standard for use in DBPCFC for evaluation of classic IgE-mediated food hypersensitivity, lyophilized fish were used for our double-blind fish challenges. In more than 11 years of performing DBPCFCs with many other food antigens, only 0.9% of DBPCFCs (5 of 573) were false-negative. Therefore the 21% false-negative reaction rate that occurred with the fish DBPCFCs suggested an unexpected lability of major fish allergens to lyophilization. Our present investigation supports the conclusion that the false-negative DBPCFCs to fish were the result of lyophilization and were not dose-related, since the positive open challenges often occurred with the first bite of the cooked fish fillet. However, with the exception of the flounder extracts on SDS-PAGE, we were unable to demonstrate this change in allergenicity of the fish extracts as a result of lyophilization by either SDS-PAGE, immunoblotting, or inhibition ELISA analyses. We think the in vitro methods performed lacked the sensitivity to detect this subtle, yet clinically relevant alteration in allergenicity. Sera from four of the patients with a false-negative DBPCFC were used in an attempt to demonstrate differences in IgE-binding. They all had a systemic component to their reaction, including one patient in whom generalized urticaria and significant wheezing with a greater than 20% fall in forced vital capacity, forced expiratory volume in 1 second and maximum midexpiratory flow developed despite early treatment with a metaproterenol metered-dose inhaler.

The 10 canned tuna extracts appeared as nearly homogenous smears on SDS-PAGE. However, allergenic epitopes could still be present in the canned tuna even though distinct bands were not obvious. This conclusion seems unlikely, however, since IgE binding to the canned tuna immunoblots was minimal to absent with sera from seven patients with a positive SPT to raw tuna extract. In addition, canned tuna by ELISA inhibition revealed decreased allergenicity compared with cooked tuna. All oral challenges with...
Salmon [5 x 10^6 ug/ml]

FIG. 6. IgE-ELISA inhibition assay of cooked versus canned salmon protein extract performed with sera from three patients with salmon allergy. Solid phase antigen is cooked salmon protein extract. Open circles represent inhibition with cooked salmon protein extract and closed circles with canned salmon protein extracts. Number on the X-axis tic is X in the salmon concentration 5 x 10^6 µg/ml (range of assay 5 pg/ml [5 x 10^-6 µg/ml] to 500 µg/ml [5 x 10^4 µg/ml]). (Cows milk whey protein extract used as negative control antigen and caused no inhibition.)

canned tuna have been negative in our 45 study patients. Children and young adults allergic to fish appear to be able to safely eat canned tuna. No foods are nonallergenic as illustrated by the very rare patient with a milk allergy reacting to a casein-hydrolysate formula. This study does not conclude that canned tuna is nonallergenic, but it does appear to be significantly less allergenic than cooked fish. Although not specifically addressed in this report, as no challenges were done with raw tuna, the in vitro data and SPT results suggest that raw tuna, which is frequently consumed in Japanese restaurants as sushi or sashimi, has the most allergenic potential and should be avoided.

Under correct Food and Drug Administration regulations, more than 20 related fish species may be labeled “tuna.” However, four species: yellow fin, skipjack, big eye, and blue-fin account for the vast bulk of tuna species used in canned tuna. This canned tuna is labeled “chunk light,” and is different from the more expensive white tuna pack, which is exclusively the albacore tuna species. In the processing of canned tuna, the whole fish is initially cooked, the skin and bones are removed, and then the cooked fish is placed in cans for retorting (pressure and steam
cooking). The fish in the final product of an institutional sized can of tuna may have been cooked for a total of 14 hours (Personal communication, 1992, Starkist, Long Beach, Calif.). Depending on the type of tuna pack, one or more of the following products is added: hydrolyzed protein (from either soy, wheat, milk, or rice), vegetable broth (made from one or more of the following: tomatoes, spinach, green or red bell pepper, potatoes, peas, parsley, beans, carrots, celery, garlic, onions), vegetable oil, water, salt, and sodium pyrophosphate. It appears that the process of canning tuna is responsible for its decreased allergenicity, rather than an inherent hypoallergenic property of the tuna itself. This conclusion is supported by the canned salmon challenge data. Two patients allergic to fresh cooked salmon could eat canned salmon without difficulty. The decreased allergenicity of canned salmon was also demonstrated by the decreased IgE-binding of sera from patients with salmon allergy to canned salmon immunoblots and by the 100 to 200 times higher concentration of canned salmon required to achieve 50% inhibition of an ELISA-inhibition assay when compared with cooked salmon.

Informing patients with milk allergy that canned tuna may contain milk protein is warranted, since several of our patients with milk allergy in this study had a history of an adverse reaction to canned tuna, but safely consumed milk-free canned tuna without incident. and a recent study confirmed the presence of significant milk protein in canned tuna. To avoid accidental ingestion of milk proteins, we recommend that patients with milk allergy use the dietetic tuna pack from a major U.S. distributor, since this tuna pack should contain no added hydrolyzed protein or vegetable broth.

In conclusion, this study suggests that processing fish may alter some of the major fish allergens responsible for IgE-mediated fish allergy. This decreased allergenicity has important clinical implications: (1) canned tuna provides a nutritious, low-fat, high protein food that can be recommended to many patients with food and fish allergies, and (2) lyophilized fish may not be appropriate for use in DBPCFCs.

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REFERENCES