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Original article

Study of the cross-reactivity of fish allergens based on a questionnaire and blood testing

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ELISA	enzyme-linked immunosorbent assay
PBS	phosphate buffered saline
PBST	PBS containing 0.05% Tween 20

ABSTRACT

Background: Parvalbumin and collagen have been identified as cross-reactive allergens for fish allergies. Although doctors realize that various fish elicit allergies, the targets of food allergen labeling laws were only mackerels and salmons in Japan and mackerels in South Korea. This study aimed to reveal the causative species for fish allergy via questionnaires and blood tests.
Methods: Questionnaire research was conducted in Japan via the internet concerning allergies for fish-allergic patients or their family members. Next, IgE reactivities and cross-reactivities of 26 fish species were analyzed using sera obtained from 16 Japanese patients who were allergic to fish parvalbumin or collagen by enzyme-linked immunosorbent assay (ELISA) and inhibition ELISA.
Results: Questionnaire research revealed that 88% patients cannot eat mackerel and salmon in addition to other fish. In addition, 85% respondents were not satisfied with the current food allergen labeling law. In ELISA analyses, we clarified that pooled serum obtained from patients with fish parvalbumin-specific allergies exhibited IgE reactivity to the extracts of most fish species, and pooled serum obtained from patients with fish collagen-specific allergies displayed IgE reactivity to the extracts of all types of fish. Inhibition ELISA experiments revealed cross-reactivities of parvalbumin or collagen to extracts from all fish tested.
Conclusions: Most patients with fish allergies displayed allergic symptoms following the intake of various fish species. In addition, fish parvalbumin and collagen were causative factors of fish allergy and were highly cross-reactive fish panallergens. Therefore, current laws should be revised in Japan and South Korea.
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Introduction

Fish is a valuable source of proteins, physiologically active substances such as eicosapentaenoic acid and docosahexaenoic acid, and minerals such as calcium. Thus, fish plays an important role in human nutrition and health. In parallel with the increase of fish consumption, however, fish allergy mediated by IgE has become a serious problem worldwide, especially in coastal countries such as Japan.

Extensive studies using the Baltic cod (*Gadus callarias*) first identified parvalbumin (called Gad c 1), a sarcoplasmic Ca^{2+}-binding protein of approximately 10 kDa, as a fish allergen.[1,2] Subsequent molecular studies revealed that parvalbumin is the major fish allergen in a number of fish, such as carp (*Cyprinus carpio*),[3] Atlantic salmon (*Salmo salar*),[4] Japanese jack mackerel (*Trachurus japonicus*),[5] crimson sea bream (*Evynnis japonica*),[6] Pacific mackerel (*Scomber japonicus*),[7] and bigeye tuna (*Thunnus obesus*).[8] Parvalbumin is produced only in vertebrates, particularly at high concentrations in fish and amphibian muscles.[9] In addition, parvalbumin is known as a major allergen, with IgE-positive rates of 67%–100%.[7,10,11] It has been revealed that various fish have common allergenicity (cross-reactivity) via parvalbumins.[12–17]

In the early 2000s, type I collagen was identified as a new second allergen for fish allergy, and approximately 30% of Japanese patients with fish allergies appear to be sensitive to fish collagens.[18,19] Collagen is a connective tissue protein, which is present in the muscles, skin, and bones in large quantities. Collagen from several species of fish has been determined to be an allergen, and

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collagens from these types of fish have cross-reactivities with each other.[20]

In Japan, with revisions to the Food Sanitation Act, the Labeling System for Foods Containing Allergens was implemented for food products in 2002. Seven ingredients must be labeled on the packages of food products as specific ingredients, and 20 ingredients should be labeled as subspecific ingredients. As per this law, only mackerels and salmons are regarded as subspecific ingredients. Moreover, the only salmon species targeted for labeling is anadromous salmon, whereas landlocked salmons are not included in the labeling. However, anadromous salmons have the same genes as landlocked salmons, and it has been reported that the allergenicity of both types of salmons is similar.[21] Furthermore, doctors realize that patients who are allergic to mackerels and/or salmons react to various species of fish. As mentioned previously, patients with fish allergies seem to exhibit allergic symptoms in response to several types of fish via cross-reactivities of parvalbumin and/or collagen.

Although several hundreds of fish species are consumed in Japan, there is no report in which the cross-reactivities of multiple fish species were investigated simultaneously in relation to both parvalbumins and collagens. Therefore, this study aimed to reveal the causative species of fish allergy via the questionnaire research. Moreover, we investigated the allergenicity and cross-reactivity of parvalbumin and collagen using 26 types of fish that are commonly consumed in Japan.

Methods

Questionnaire research

Questionnaire research was anonymously conducted for fish-allergic patients or their family members through the internet. The implementation period was from May 2014 to July 2015. In total, 97 responses to the questionnaire, including 95 valid responses, were received.

Samples

The following 26 species of fish, which are widely consumed in Japan, were used as samples: Round herring (*Etrumeus teres*), Pacific herring (*Clupea pallasii*), Japanese sardine (*Sardinops melanostictus*), chum salmon (*Oncorhynchus keta*), silver salmon (*Oncorhynchus kisutch*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*S. salar*), Pacific cod (*Gadus macrocephalus*), splendid alfonsino (*Beryx splendens*), Mediterranean flying fish (*Cheilopogon heterurus*), Pacific saury (*Cololabis saira*), goldeye rockfish (*Sebastes thompsoni*), rosy seabass (*Doederleinia berycoides*), Japanese amberjack (*Seriola quinqueradiata*), Japanese jack mackerel (*T. japonicus*), chicken grunt (*Parapristipoma trilineatum*), red seabream (*Pagrus major*), white croaker (*Pennahia argentata*), swordfish (*Xiphias gladius*), red barracuda (*Sphyraena pinguis*), skipjack (*Katsuwonus pelamis*), Japanese Spanish mackerel (*Scomberomorus niphonius*), Pacific mackerel (*S. japonicus*), blue mackerel (*Scomber australasicus*), bigeye tuna (*T. obesus*), and yellowfin tuna (*Thunnus albacares*). Fresh filet samples (for 10 species of fish, including chum salmon, silver salmon, Atlantic salmon, Pacific cod, Japanese amberjack, swordfish, skipjack, Japanese Spanish mackerel, bigeye tuna, and yellowfin tuna) or round fish samples (for the remaining 16 species of fish) were purchased from local retail shops in Tokyo and immediately subjected to experiments. Round herring, chum salmon, white croaker, and bigeye tuna were used only for the investigation of parvalbumin, and Pacific herring, Pacific cod, Japanese amberjack, and Japanese Spanish mackerel were used only for the investigation of collagen. In addition to fish, third-stage larvae of *Anisakis simplex* with cysts were collected from the hepatopancreas surface of the Alaska pollack (*Gadus chalcogrammus*). After the cysts were digested with pepsin according to the method of Bier *et al.*,[22] the larvae were washed with a 0.9% NaCl solution and stored at −20 °C until use.

Preparation of extracts

In case of fillets, skin, bones, and dark muscles were removed from the fillets. Only white muscles were individually minced well, and a part of each minced sample was used for experiments. In case of round fish, the head, fins, skin, bones, whole organs, and dark muscles were removed, and only whole white muscles of each fish were used as described above for the experiments. For the analysis of parvalbumin, the following extract was used: the mince of white muscle collected from each specimen was homogenized in four volumes of 150 mM NaCl-10 mM phosphate buffer [pH 7.0; phosphate buffered saline (PBS)]. After heating at 100 °C for 10 min, the homogenate was centrifuged at 16,000 ×*g* for 5 min, and the obtained supernatant was used for experiments. The extracts were stored at −20 °C until further use. For the analysis of collagen, the following extract was used: the mince of white muscle collected from each specimen was homogenized in four volumes of 50 mM Tris-HCl (pH 8.0) containing 7 M urea and 2 M thiourea. The homogenate was centrifuged at 16,000 ×*g* for 5 min, and the obtained supernatant was used for experiments. The extracts were diluted 10-fold with the same buffer and immediately subjected to experiments without freezing.

For the preparation of an extract of *A. simplex*, the larvae were extracted with four volumes of PBS. After centrifugation at 16,000 ×*g* for 5 min, the supernatant obtained was used as a crude extract. The extract was stored at −20 °C until use.

Purification of parvalbumin and collagen

Parvalbumin was purified from the white muscle of Pacific mackerel according to the method of Shiomi *et al.*[8] Collagen was purified from the skin of Pacific mackerel according to the method of Miller and Rhodes.[23] The concentration of each allergen was determined according to the method of Lowry *et al.*[24] using bovine serum albumin as a standard.

Human sera

Sera were obtained from 16 fish-allergic patients with documented clinical histories of immediate hypersensitivity reactions after the ingestion of fish. All the patients were Japanese. Written informed consent was obtained from each patient, and patient anonymity was preserved. First, all patients were checked by medical doctors and were diagnosed to be allergic to fish. Next, all patients were tested using ImmunoCAP (Phadia, Uppsala, Sweden), and the classes for some fish extracts were two to six. In addition to this, skin prick testing was done, and all the patients gave positive results for various fish species. IgE reactivities of patients' sera to Pacific mackerel parvalbumin or collagen were checked by enzyme-linked immunosorbent assay (ELISA). Sera from patients with parvalbumin-specific allergy (n = 8) and collagen-specific allergy (n = 8) were used for the following experiments. In the present study, pooled serum from 10 healthy volunteers (Cosmo Bio, Tokyo, Japan) was used as a control. All experiments using human sera were performed following the Ethical Guidelines of Tokyo University of Marine Science and Technology (Permit Number: 26-002). The study was conducted in accordance with the principles embodied in the Declaration of Helsinki.

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