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Recidivous acute urticaria caused by *Anisakis simplex*

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This study aimed to determine the cause of acute recidivous urticaria in patients who usually eat fish or other seafood. Twenty-five patients were studied. The skin prick test with larval *Anisakis simplex* extract was performed; total and specific IgE against *A. simplex* was measured with the CAP System; specific antibodies to *A. simplex* were determined by ELISA; and immunorecognition patterns of the sera were studied by Western blot. Nineteen patients showed specific IgE to *A. simplex*, but specific IgE to *Ascaris* was demonstrated in only two patients. No patients reacted to *Toxocara canis* or *Echinoccocus granulosus* antigens with the same test. The skin prick test was positive in 16 patients, in two of them persisting for 48 h. Five patients showed neither skin reaction nor specific IgE to *A. simplex*. Sera showed specific immunoglobulin levels against *A. simplex* larval crude extract, by both ELISA and Western blot. Likewise, specific immunoglobulin levels against excretory-secretory antigen were also measured by ELISA. Only one patient showed sensitization to fish. *A. simplex* was found to be the main cause of acute recidivous urticaria in patients who usually eat fish and are not sensitized to it.

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Urticaria is a very common allergic disease with a cumulative prevalence of 15–25%. In approximately 50% of the cases, hives are associated with angioedema presenting as swelling of the subcutaneous tissues. Most cases of chronic and recurrent acute urticaria have been labeled idiopathic. In reports in which extensive evaluation has been performed, no causative factor could be found in up to 70% of cases. The identification of any orally ingested agent as a cause of urticaria may at times be difficult. Occult, clinically relevant allergens may be present in a patient's diet. We think that this could explain the urticaria elicited by fish infected with *Anisakis simplex* larvae.

Anisakidosis is a human disease caused by the ingestion of larval nematodes of the family Anisakidae, especially *A. simplex*. The infestation is acquired by eating raw seafood or undercooked fish and squid (1). Anisakidosis is divided into gastric, intestinal, and ectopic (2). Due to the vagueness of its symptoms, this disease is often misdiagnosed as appendicitis, acute abdomen, gastric tumor or cancer, ileitis, cholecystitis, diverticulitis, tuberculous peritonitis, cancer of the pancreas, or Crohn's disease (1).

The first case of human anisakidosis in Spain was reported by Arenal et al. (3). It was a case of acute abdomen due to appendicular lumen occlusion by *Anisakis* larvae. Latterly, López-Vélez et al. (4) have reported three cases of nonimported anisakidosis in Spanish patients. In two of them, the symptom was bowel obstruction, and a larva of *A. simplex* was found by intestinal biopsy in the abdominal wall. In the third case, presenting as peptic ulcer disease with longer evolution time, a larva of *Pseudoterranova decipiens* was found during an upper gastrointestinal endoscopic procedure. Likewise, Valero et al. (5) identified larvae of *A. simplex* after surgical intervention in a patient with clinical indication of peritonitis. Finally, Clavel et al. (6) reported a case of anisakidosis in which a live nematode larva was found in the abdominal cavity of a woman. It was identified as *A. physeteris*.

Acute symptoms of anisakidosis may be caused by a type I allergic reaction in the gastrointestinal wall (7, 8) with elevated specific IgE after onset of clinical symptoms (9). On the other hand, Kasuya et al. (10) observed that *Anisakis* larvae were the real causative agent in some patients with urticaria

but without abdominal pain and, therefore, with no clinical indication of anisakidosis.

This work aimed to determine the cause of recidivous acute urticaria in 25 patients who usually ate fish and/or other seafood.

Material and methods

Patients and serum samples

Twenty-five patients with unknown cause recidivous acute urticaria were selected for this study (16 females; nine males; age range: 39.26±19.82 years, minimum: 11, maximum: 77). They visited the immunology and allergy service of the Hospital del Aire (Madrid, Spain) in September and October 1995. They all lived in Madrid.

These patients ate fish regularly. They were diagnosed as having idiopathic acute recidivous urticaria. Patients diagnosed with any other allergic disease were excluded, as were patients taking corticosteroids or antihistaminics. All patients underwent the usual protocol related to idiopathic acute recidivous urticaria (to exclude other trigger factors): complete blood count, biochemistry (urea, glucose, cholesterol, GOT, GGT, total proteins, seric Fe, albumin, alkaline phosphatase, LDH, and amylase), urine, C3, C4, C1q, total hemolytic complement, fecal parasites, hepatitis B and C, and skin prick test (inhaled and food-derived allergens). The intervals of symptoms developed ranged from 2 months to 13 years.

Human serum E17 (anisakidosis reference serum) was supplied by the Contrôle National de Qualité en Parasitologie (Département de Biologie Medicale E. Brumpt, Centre Hospitalier, 95500 Gonesse, France). The E17 reference serum was obtained 2 months after infection and tested by Western blot, immunoelectrophoresis, and the Ouchterlony test (11). As negative controls, human sera previously selected by ELISA and Western blot were used (12).

Antigen preparation

Third-stage larvae (L3) of *A. simplex* were extracted manually from the viscera, flesh, and body cavity of naturally infected hake (*Merluccius merluccius*) and thoroughly washed in water. For the preparation of the crude extract (CE), *A. simplex* L3 were placed at 4°C in PBS buffer. This material was homogenized in a hand-operated glass tissue grinder at 4°C, after sonication for 6 min (10 s/pulse) with a Virsonic 5 (Virtis) set at 70% output power. The homogenate was extracted in PBS at 4°C overnight and subsequently delipidized with *n*-hexane and then centrifuged at 8497 *g* for 30 min at 4°C (Biofuge 17RS, Heraeus Sepatech). The supernatant was dialyzed overnight at 4°C in PBS, and its protein content was estimated by the Bradford method (13). The extract was frozen at –20°C until use (14).

To obtain excretory-secretory (ES) products, viable *A. simplex* larvae were carefully selected, and immersed for 1 h in NaCl (0.85%) containing gentamicin (4 g/l). One larva per ml was placed in each well of 24-well plates (COSTAR, Cambridge, MA, USA) containing 1 ml of Eagle's minimum essential medium with Earle's salts (ICN Biomedicals, Inc., Costa Mesa, CA, USA) supplemented with sodium bicarbonate (2.2 g/l), glutamine (0.292 g/l), and gentamicin (4 g/l), and maintained at 28°C. Supernatants were collected weekly. After dialyzing against PBS (0.01 M sodium phosphate; 0.15 M NaCl) and concentration in an Amicon YM-10, the protein content in the supernatant was estimated by the Bradford method (13) and finally frozen at –20°C until use (14).

Enzyme-linked immunosorbent assay (ELISA)

The 96-well microtiter plates (Nunc-Immuno Plate MaxiSorp™) were sensitized overnight at 4°C by the addition of 1 μg/ml of ES or larval CE antigen diluted in a carbonate buffer to 0.1 M at pH 9.6. After washing three times with 0.05% PBS-Tween 20 (PBS-Tween), wells were blocked by the addition of 200 μl per well of 0.1% BSA in PBS, for 1 h at 37°C. After washing, 100 μl of duplicate dilutions of sera at 1/100, in PBS-Tween, containing 0.1% BSA, was added and incubated at 37°C for 2 h. Once the plates were washed, 100 μl per well of affinity-isolated, peroxidase-conjugated goat antihuman immunoglobulins (gamma-, alpha-, mu-, and light-chains) (Tago, Inc.), at the appropriate dilution in PBS-Tween, 0.1% BSA, was incubated for 1 h at 37°C. After washing, 100 μl per well of substrate (*o*-phenylene-diamine; Sigma) was added at 0.04% in phosphate-citrate buffer (pH 5.0) with 0.04% hydrogen peroxide. The reaction was stopped with 3 N sulfuric acid, and the plates were read at 492 nm. Simultaneously, an ELISA without antigen was provided as control. For evaluation of the results obtained from human sera, the diagnostic index (DI) was used, as the ratio between the optical density (OD) resulting from the test serum and the OD of the negative control once its corresponding nonspecific reaction with the BSA used in the postcoating was subtracted (12, 15).

Immunoblotting analysis

Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) was carried out, as described

by Laemmli (16) and revised by Hames (1986) (17), with a Mini Protean® II cell (Bio-Rad). The gels consisted of a 4% stacking gel and a 5–20% linear gradient separating gel. Larval CE antigen was dissolved in a sample buffer (50 mM Tris-HCl buffer, pH 8.6, containing 2% SDS, 20% glycerol, and 0.02% bromophenol blue) diluted 1 : 1 in electrode buffer (25 mM Tris, 192 mM glycine, pH 8.3), containing 1% SDS. Electrophoresis was performed for 2 h at a constant 100 V in Tris-glycine electrode buffer (see above). Broad-range molecular weight markers (6500–205 000 Da, Bio-Rad) were incorporated into each electrophoretic run.

After the SDS–PAGE of the larval CE antigen, the protein bands were transferred onto a 0.22-μm pore size nitrocellulose membrane (Pharmacia) in a Multiphor II Nova Blot semidry transfer cell (Pharmacia) with 48 mM Tris, 39 mM glycine, 0.037% SDS, and 20% methanol, pH 9.2. The transblot was carried out at 45 mA constant current for 12 h. The membrane was cut into strips and each strip was blocked for 3 h at room temperature with PBS containing 5% nonfat dry milk, before immunorecognition by incubation for 3 h with the sera diluted at 1/50 in PBS-Tween containing 1% nonfat dry milk. Each strip was then washed with PBS-Tween (3×5 min) and incubated for 3 h with affinity-isolated, peroxidase-conjugated goat anti-human immunoglobulins (gamma-, alpha-, mu-, and light-chains) (Tago, Inc.), at the appropriate dilution in PBS-Tween containing 1% nonfat dry milk. For visualization of bands, the strips were washed with PBS-Tween (3×5 min) and reacted with the substrate (PBS, containing 0.006% H_2O_2 : methanol, containing 0.03% 4-chloro-1-naphthol). The reaction was stopped by thorough washing with distilled water, and the stained bands were photographed (12).

Skin test

A. simplex extract (International Pharmaceutical Immunology from ASAC, Pharmaceutical International, AIE) containing 1 mg/ml was tested on the volar forearm skin by the prick method. Reactions were considered to be positive when a wheal diameter of 3 mm was present 15 min later. Histamine (10 mg/ml) and saline solution were used as positive and negative controls, respectively. Skin tests to white fish (sea bream [*Pagellus centrodontus*], anchovy [*Engraulis encrasicolus*], red mullet [*Mullus surmuletus*], and pilchard [*Sardina pilchardus*]); blue fish (cod [*Gadus morrhua*], common sole [*Solea solea*], common bass [*Roccus labras*], and hake [*M. merluccius*]); and *A. simplex* were performed (Pharmacia Diagnostics).

Determination of total IgE

In vitro assays were done with the Pharmacia CAP System IgE FEIA/TEMPO (Pharmacia & Upjohn, Uppsala, Sweden).

Determination of specific IgE

In vitro assays were done with the Pharmacia CAP System FEIA (Pharmacia & Upjohn, Uppsala, Sweden) for antigen-specific IgE against *Anisakis*, *Ascaris*, *Echinococcus*, *Toxocara*, tuna fish, salmon, shrimp, mussel, and cod. According to the manufacturer's instructions, results greater than 0.35 kU/l should be considered positive (CAP=1 or more). But in an attempt to avoid cross-reaction or false-positives, only results greater than 0.70 kU/l were considered positive (CAP=2 or more).

Statistical analysis

The Mann-Whitney test was used to study significant differences between the *A. simplex* skin test and *A. simplex* CAP System values. Correlation coefficients were also determined by the Spearman test.

Results

We examined a total of 25 patients with recidivous acute urticaria due to an unknown cause. These patients developed urticaria after ingestion of fish and/or other seafood. The interval between eating fish or other seafood and developing urticaria and/or angioedema was 3–36 h. The *Anisakis* skin prick test was positive in 16 patients, two of whom developed delayed reactions (24–48 h). The fish skin test was negative in all but one patient (no. 5), who also showed positive reaction by CAP to salmon (1.38 kU/l), shrimp (0.715 kU/l), and mussel (0.753 kU/l). The *Anisakis* skin prick test and fish (white and blue fish) skin test were negative. It must be taken into account that salmon is not included in the mixed blue fish skin prick test. It must also be noted that this 19-year-old woman was CAP-positive to gramineous pollen (*Lolium perenne* and *Phleum pratense*), to plant pollen (*Plantago lanceolata*, *Chenopodium album*, and *Salsola cali*), to tree pollen (*Ulmus americana*, *Platanus acerifolia*, *Fraxinus americana*, and *Acacia longifolia*), and to several food types: fish, vegetables, fruits, etc. (salmon, shrimp, mussel, tomato, peach, peanut, hazelnut, chickpea, black pepper, etc.).

Total IgE was higher than 120 kU/l in 15 patients, but lower in the other 10 (total IgE mean: 362.68 ±113.66; minimum: 9.7, maximum: 2000). In 19 of

the 25 cases, specific IgE to *A. simplex* with the Pharmacia CAP System FEIA was shown (specific IgE to *Anisakis*, mean: 7.26±3.02; minimum: 0.35, maximum: 71.4). Specific IgE against *Ascaris* was demonstrated in only two cases (patients 8 and 19).

Table 1. Results of serologic and allergologic tests on selected patients

Patients	Prick test	RAST (class)	RAST (kU/l)	Total IgE (kU/l)
1	+	3	4.29	22.9
2	+	3	14.20	47.3
3	+	3	5.43	635
4	-	2	1.30	9.70
5	-	2	0.96	170
6	-	2	2.22	1401
7	–	2	1.57	21
8	+	2	2.00	2000
9	+	2	1.28	49.7
10	+	2	0.79	32.5
11	+	2	1.57	354
12	+	2	2.07	30
13	+	2	3.36	110
14	–	1	0.61	316
15	+	3	6.20	730
16	+	1	0.60	915
17	+	3	14.20	7.38
18	+	3	10.50	164
19	+	5	71.40	1223
20	-	0	0.35	491
21	+	2	1.50	146
22	+	4	29.80	188
23	–	0	0.35	20.2
24	-	0	0.35	208
25	–	0	0.35	164

RAST class: according to Pharmacia Cap System RAST™ FEIA; DI: ratio between OD from serum and OD of negative control once its corresponding nonspecific reaction with BSA used in postcoating was subtracted; T: *A. simplex* crude extract; ES: *A. simplex* excretory-secretory products.

No patient reacted to *Toxocara* or *Echinococcus* antigens with the same test. Clinical manifestations were developed by patients, and test results are shown in Table 1. Specific immunoglobulin levels against *A. simplex* larvae crude extract and ES products were determined by ELISA. The following results were obtained: crude extract mean DI: 1.23±0.26 (maximum: 1.88; minimum: 0.88); ES products mean: 1.40±0.31 (maximum: 2.19; minimum: 0.88). All of the above results are shown in Fig. 1

Immunorecognition patterns of these sera are shown in Fig. 2. Fourteen of the 25 tested sera showed the characteristic band at 49.8–80 kDa compared to the E17 reference serum. Most of the sera showed a common immunorecognition pattern with a group of bands at 200–80 kDa. Only six sera recognized proteins of low molecular weight.

Discussion

In this study, 25 patients with recidivous acute urticaria were studied. The work aimed to investigate the possible cause of the recidivous acute urticaria in these patients. In 19 of the 25 cases, specific IgE to *Anisakis* was shown. Kasuya et al. (10) suggested that sea-fish-induced urticaria may be an allergic response to *Anisakis* larval antigen rather than to the fish itself. Therefore, skin sensitivity anti-IgE tests to fish (white and blue fish) were undertaken in all patients. Moreover, the first case of periodic anaphylactic episodes to *A. simplex* in Spain, reported by Audícana et al. (18), was confirmed by prick test and specific IgE detection.

Only one patient (no. 5) showed both *Anisakis* anti-IgE (0.958 kU/l) and fish anti-IgE (salmon,

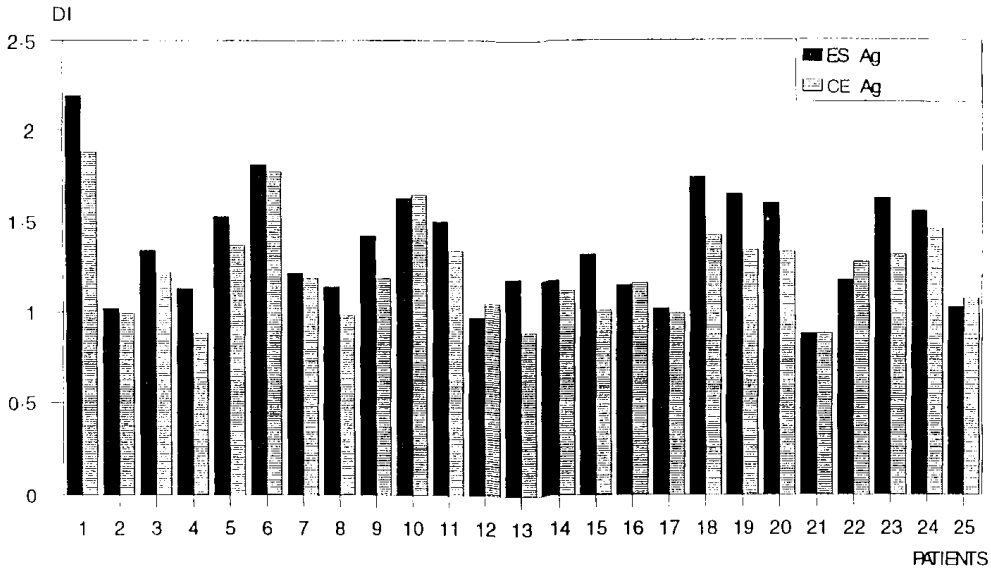

Fig. 1. Immunoresponses in sera from selected patients against *A. simplex* CE and ES antigens by ELISA. See figure for explanation of bars. DI: diagnostic index.

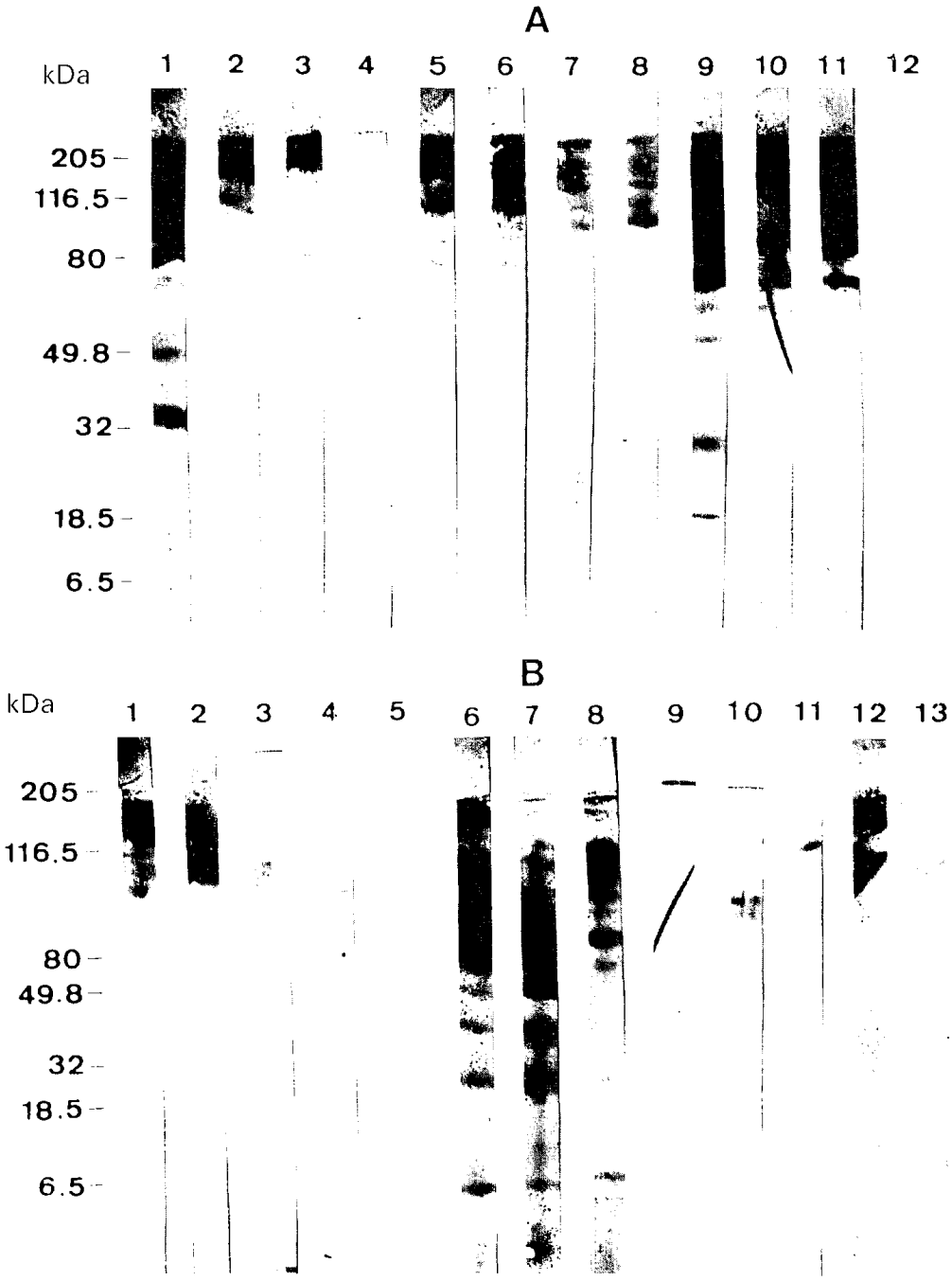

Fig. 2. Immunorecognition patterns against CE antigen of *A. simplex* under nonreducing conditions of human sera. A) Strip-serum: 1-1; 2-2; 3-3; 4-4; 5-5; 6-6; 7-7; 8-8; 9-9; 10-10; 11-11; 12-12. B) Strip-serum 1-13; 2-14; 3-15; 4-16; 5-20; 6-18; 7-19; 8-17; 9-24; 10-22; 11-23; 12-21; 13-25.

1.38 kU/l; shrimp, 0.715 kU/l; and mussel, 0.753 kU/l).

The *Anisakis* skin prick test was positive in 16 patients, while *Anisakis*-specific anti-IgE was positive in 19. Significant differences were observed between the *A. simplex* skin test and *A. simplex* CAP System values (P=0.016) when the Mann-Whitney test was used.

Sixteen patients showed a positive skin prick test to *A. simplex* extract. Acute symptoms of anisakidosis could be caused by a type I allergic reaction in the gastrointestinal wall with elevated specific IgE after onset of clinical symptoms (7, 8). Otherwise, two of these 16 patients showed a maintained papule 48–72 h after the skin prick test was performed. This fact suggests that this antigen may be able to produce delayed-type hypersensitivity reactions.

Likewise, specific IgE against *Ascaris* was demonstrated in two cases. *Ascaris*-specific anti-IgE levels were similar to or lower than *Anisakis* anti-IgE levels in all but one case. There is known cross-reactivity between *Ascaris* and *Anisakis* antigens. Sakanari et al. (19) suggested that there were shared epitopes between these taxonomically related nematodes. This fact could be problematic

if the patient has had previous *Ascaris*-positive infection. This problem can be avoided by studying each serum immunorecognition pattern, a positive anisakidosis reference serum being used as positive control.

Nevertheless, the greatest variety of evidence must be assembled, as an anamnesis, in order to have sufficient data to establish a diagnosis. The future use of *Anisakis*-specific monoclonal antibodies or recombinant antigens could eliminate this problem.

The ELISA data showed IgG, IgM, and IgA levels to *Anisakis* larval antigen. By Spearman's correlation coefficient, no linear correlation between IgE levels and IgG, IgM, and IgA levels was observed. This fact could suggest different immune responses depending on the nature of the illness (gastric and intestinal *vs* sensitization to *Anisakis*). *A. simplex* sensitization may have been caused by a previous larval infestation or by continuous contact with parasite protein, but not by ingestion of viable larvae.

One of our patients, an 11-year-old girl (patient no. 11), was diagnosed as having an obstructive intestinal illness which climaxed with abdominal pain and fever (39.5°C). One month after the symptoms had disappeared, the patient was sent to our allergology department to be examined for possible allergy to drugs (family antecedents: allergic mother). The patient proved to be negative to drugs but positive to *Anisakis* (1.75 kU/l). Her mother (patient no. 10) was also investigated because she had presented mild gastric symptoms. Both of them had eaten fish and the mother proved to be *Anisakis*-positive as well. Both patients showed high levels of anti-*Anisakis*-specific antibody, suggesting intestinal anisakidosis.

Conversely, high CAP System specific IgE titers with low anti-*Anisakis* antibodies by ELISA titers could detect an allergic reaction due to *Anisakis* somatic products from fish without viable *Anisakis* larvae ingestion.

Some countries have regulations to prevent anisakidosis. In 1967, The Netherlands passed a law requiring fresh herring to be frozen at at least –20°C for 24 h. In 1987, the US Food and Drug Administration also established regulations (freezing or boiling of fish) to prevent this problem (20). Such a regulation prevents most intestinal and gastric anisakidosis cases, but what about allergy to dead *Anisakis* larvae?

The thermostability of *Anisakis* allergens has been demonstrated by prick skin test with the heated crude extract (18); therefore, ingestion of cooked fish does not prevent allergic manifestations. A similar problem could be presented after freezing. On the other hand, one of our patients (no. 12), a 70-year-old woman who was *Anisakis*-positive (2.07 kU/l) and had developed facial recidivous angioedema, was advised not to eat any fish. Her symptoms improved until she ate pizza made with canned tuna. The problem now is that raw or fresh fish may cause gastric/intestinal/ectopic/allergic anisakidosis, but cooked, frozen, and even canned fish can also cause allergic manifestations.

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