

Immediate and Cell-Mediated Reactions in Parasitic Infections by *Anisakis simplex*

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■ Abstract

Background: *Anisakis simplex* is responsible for allergic symptoms after repeated ingestion or contact with parasitized fish.

Objective: To further analyze type I and IV immunologic mechanisms in a group of patients with allergic reactions to *A simplex*, we performed prick-by-prick testing with *A simplex* larvae and patch tests with live, cooked, or frozen larvae of the *A simplex* parasite.

Methods: Thirty-eight patients underwent skin prick test and radioallergosorbent test with inhalant allergens, foods, and *A simplex*. Prick-by-prick tests with *A simplex* and patch tests with live, cooked, and frozen larvae were carried out in 10 patients with evidence of allergy to *A simplex*.

Results: Prick-by-prick testing yielded a positive result in 100% of cases with live larvae and in 70% with cooked and frozen larvae. Patch tests with *A simplex* were positive in 8 patients for live larvae, in 3 patients for frozen larvae, and in 1 patient for cooked larvae.

Conclusion: Our data emphasize that *A simplex* is responsible for both immediate allergic reactions and cell-mediated (delayed) reactions, in particular in subjects with occupational exposure. In addition, our data demonstrate that not only live but also frozen and cooked larvae can induce sensitization. This observation may be explained by differences in the allergenic proteins involved, and further studies will be required to address this possibility.

Key words: Allergic contact dermatitis. *Anisakis simplex*. Patch test. Specific immunoglobulin E. Skin prick test.

■ Resumen

Antecedentes: El *Anisakis simplex* es el causante de síntomas alérgicos después de reiteradas ingestiones o contacto con peces parasitados.

Objetivo: Para analizar en profundidad los mecanismos inmunológicos de tipo I y IV en un grupo de pacientes con reacciones alérgicas al *A simplex* realizamos pruebas de prick-by-prick con larvas de *A simplex* y pruebas epicutáneas con larvas del parásito *A simplex* vivas, cocinadas o congeladas.

Métodos: Se practicó una prueba cutánea y una prueba de radioalergoadsorción con alérgenos inhalantes, alimentos y *A simplex* a 38 pacientes. Se sometió a 10 pacientes con una alergia a *A simplex* evidente a las pruebas de prick-by-prick con *A simplex* y a las pruebas epicutáneas con larvas vivas, cocinadas y congeladas.

Resultados: Las pruebas de prick-by-prick dieron un resultado positivo en el 100% de los casos en los que se habían utilizado larvas vivas y en el 70% de los casos en los que se habían usado larvas cocinadas y congeladas. Las pruebas epicutáneas con *A simplex* fueron positivas en 8 pacientes para las larvas vivas, en 3 para las larvas congeladas y en 1 para las larvas cocinadas.

Conclusión: Nuestros datos destacan que el *A simplex* es el causante tanto de reacciones alérgicas inmediatas, como de reacciones (retardadas) mediadas por células, en especial en aquellos sujetos con exposición laboral al *A simplex*. Además, nuestros resultados demuestran que no sólo las larvas vivas pueden inducir una sensibilización, sino también las congeladas y las cocinadas. Esta observación se puede explicar por las diferencias en las proteínas alérgicas implicadas y son necesarios estudios adicionales para abordar esta posibilidad.

Palabras clave: Dermatitis alérgica por contacto. *Anisakis simplex*. Prueba epicutánea. Inmunoglobulina E específica. Prueba cutánea.

Introduction

Anisakis simplex is a marine nematode belonging to the order Ascaridida, family Heterocheillidae. It is found throughout the world, and its life cycle is characterized by several stages in which it parasitizes marine animal species. Many teleost fish and cephalopods contain third-stage larvae in the celomic cavity and viscera, and from these organs the larvae can reach the muscles (Figure 1). The larvae are white and measure 2 to 4 cm in length. The parasitized fish species most often consumed by humans are cod, hake, anchovies, salmon, sardines, tuna, red mullet, and mackerel. Following ingestion, the larvae can penetrate the human gastric or intestinal mucosa by releasing proteases, leading to gastric or intestinal parasitic infection (anisakiasis).



Figure 1. Larvae of *Anisakis simplex* in the celomic cavity of a fish.

The first report of gastric anisakiasis was made by van Thiel [1] in 1960, and to date, the allergic importance of *A simplex* has been underestimated. Ingestion or repeated contact with fish parasitized by stage 3 *A simplex* larvae (L3) can provoke urticaria-angioedema syndrome [2] and anaphylactic shock [3]. In the literature, only 1 study has described allergic contact dermatitis (2 cases) involving a cell-mediated immune reaction triggered by direct contact with raw parasitized fish [4]. That study suggested that eczematous lesions could be directly induced by contact with the nematode rather than food consumption in subjects who frequently handle fish as part of their job (workers in fish farms, fishmongers, cooks, etc). In light of these observations, the aim of the present study was to analyze type I sensitization in a group of patients with allergic reactions to fish. In addition, we analyzed cell-mediated reactions characterized by eczematous symptoms and the results of patch tests with the nematode larvae in a subgroup of patients with evidence of allergy to *A simplex*.

Materials and Methods

Patients

In the period between October 2005 and June 2006, we observed 38 patients with allergic reactions to fish. The group included 25 women and 13 men aged between 18 and 78 years, with a mean age of 46.7 years. Twenty-three patients had a family history of atopy. All of them had repeated occupational contact with fish or reported frequent consumption of raw fish. During the clinical examination, we inquired about the type of food eaten, the time of onset of symptoms after ingesting seafood, and the way in which the food was cooked or prepared.

Prick-by-prick test and patch testing with *Anisakis* larvae was only performed in patients who met the following criteria: a positive skin prick test (SPT) to *A simplex* and negative results for other food allergens; positive results for *A simplex* and negative results for other allergens in radioallergosorbent test (RAST); negative results after application of a standard diagnostic patch test panel; and agreement to undergo the prick-by-prick testing and patch testing with the “*Anisakis* series.” Among these patients, 6 had occupational contact with fish and 4 were homemakers. In all 10 patients, the possibility of contamination with protein from the host fish was excluded since negative results were obtained for SPT and RAST with other fish allergens. The control group consisted of 12 healthy volunteers (8 women and 4 men aged between 25 and 60 years; mean age, 42.7 years), none of whom were atopic subjects.

All participants gave informed consent to testing.

Larvae Preparation Method

Six hundred *A simplex* larvae were extracted from hake and separate into 3 groups: (1) 200 worms were cooked for 10 minutes at 100°C; (2) 200 were stored for 48 hours at -24°C; and (3) 200 were kept alive at 2°C to 4°C until use. Larvae were prepared according to the method reported by Conde-Salazar et al [4], who described 2 cases of hand eczema after contact with raw fish, and based on the European Directive of 1998 on food safety [5] and US Food and Drugs Administration legislation [6]. In particular, those recommendations indicate that fish should be frozen at -20°C for 7 days or at -15°C for 15 hours before consumption. After patch-test preparation, the remaining worms (live, cooked, and frozen) were put in 3 different boxes and used for prick-by-prick tests.

Skin Prick Test

A positive control (histamine, 10 mg/L) and a negative control (glycerinated saline) were used along with a panel of food allergens (tuna, salmon, mackerel, sardine, shrimp, and octopus), inhalants (*Dermatophagoides pteronyssinus*, *Acarus siro*, *Lepidoglyphus destructor*, and *Tyrophagus putrescentiae*), and *A simplex* (Bayer, Milan, Italy). Test results were interpreted according to the guidelines of the European Academy of Allergology and Clinical Immunology-Subcommittee on Allergen Standardization and Skin Tests [7]. If the diameter of the reaction was more than 3 mm greater than the one provoked by the negative control, the response was considered positive.

RAST

RAST was assessed to detect specific immunoglobulin (Ig) E (UniCAP system, Pharmacia, Sweden) against *A simplex*, food allergens (anchovy, lobster, herring, shrimp, crab, swordfish, octopus, cuttlefish, cod, shellfish, mackerel, sardine, tuna, and salmon), inhalant allergens (*D pteronyssinus*, *A siro*, *L destructor*, and *T putrescentia*), and nematodes (*Ascaris lumbricoides* and *Echinococcus granulosus*). Results were considered positive for specific IgE if the concentration was greater than 0.35 kU_A/L [8].

Prick-by-Prick Test

The prick-by-prick test represents a second-level test

used for diagnosis of IgE-mediated food allergy. The test was performed using L3 *A simplex* larvae by first introducing a sterile lancet into the fresh food (in this case into the box containing *A simplex* larvae) and then into the skin of the patient's forearm. The results of prick-by-prick were evaluated as for SPT. It is important to stress that this method does not give rise to infectious complications.

Patch Testing With the *Anisakis* Series

Patch tests with the *Anisakis* series were applied to the skin on the patient's back, removed after 48 hours, and interpreted at 48 hours and 96 hours. Briefly, a polyethylene support (IQ chambers, Chemotechnique, Vellinge, Sweden) containing

Table. Symptoms and Test Results in 10 patients With Suspected *Anisakis* Sensitization

Patients	Suspected Food	Method of Preparation	Nausea and Vomiting	Pruritus	Urticaria Angioedema	Anaphylactic Shock	Eczema	SPT <i>Anisakis</i>	Specific IgE Against <i>Anisakis</i> , kU _A /L	Prick-by-Prick With Larvae	Patch Test, <i>Anisakis</i> series
1	Shellfish Sardines	Raw	No	Yes	Yes	No	No	++	>100	Cooked + Live ++ Frozen +	Cooked - Live +++ Frozen ++
2	Sardines	Marinated	No	Yes	No	Yes	Yes	+	2.47	Cooked - Live + Frozen ++	Cooked - Live+++ Frozen ++
3	Sardines	Cooked	Yes	Yes	Yes	No	Yes	+++	>100	Cooked +++ Live ++ Frozen ++	Cooked ++ Live++ Frozen -
4	Sardines	Marinated	No	Yes	Yes	No	Yes	++	65.4	Cooked + Live ++ Frozen +	Cooked - Live+++ Frozen -
5	Sardines	Raw	No	No	Yes	Yes	No	++	5.40	Cooked - Live + Frozen -	Cooked - Live- Frozen -
6	Shellfish Sardines	Raw	No	Yes	Yes	Yes	No	+++	12.2	Cooked + Live ++ Frozen +	Cooked - Live ++ Frozen -
7	Mackerel	Cooked	No	Yes	Yes	No	No	+++	5.58	Cooked ++ Live + Frozen -	Cooked - Live - Frozen -
8	Sardines	Raw	No	Yes	Yes	No	No	++	56.8	Cooked +++ Live ++ Frozen +	Cooked - Live + Frozen ++
9	Mackerel Sardines	Cooked	No	Yes	Yes	No	No	++	3.11	Cooked +++ Live ++ Frozen -	Cooked - Live +++ Frozen +
10	Mackerel Sardines	Raw	No	Yes	Yes	No	Yes	+	6.47	Cooked - Live +++ Frozen ++	Cooked - Live ++ Frozen ++

Abbreviation: IgE, immunoglobulin E; SPT, skin prick test.

4 wells was used. In the first we put 15 live larvae, in the second 15 cooked larvae, in the third 15 frozen larvae, and finally, in the fourth, petrolatum as negative control. Results were evaluated according to International Contact Dermatitis Research Group recommendations [9].

Results

All patients reported having suffered diffuse itching, urticaria or urticaria-angioedema syndrome, or episodes of anaphylactic shock, with or without gastrointestinal symptoms (nausea, vomiting, diarrhea, and abdominal or epigastric pain) within 24 hours of ingestion of fish, crustaceans, or cephalopods. Some of the patients suffered from intense itching and eczematous hand lesions after repeated handling of raw fish.

Symptoms were associated with sardines in 17 patients, shellfish in 8, mackerel in 7, shrimp in 5, cod in 3, and salmon in 1. In 8 patients it was not possible to identify the food responsible for the allergic symptoms. Thirteen patients had ingested cooked fish, 10 raw fish, 5 fish marinated in lemon or vinegar, 1 smoked fish, and finally, 1 patient had eaten canned fish.

The patients reported diffuse itching in 86.8% cases (33 patients), urticaria-angioedema syndrome in 73.7% (28 patients), anaphylactic shock in 26.3% (10 patients), and eczema after contact with raw fish in 6 patients (15.8%); in addition, nausea and vomiting were reported by 5 patients (13.1%) and 4 patients (10.5%) complained of epigastralgia. In 5 patients, gastrointestinal and allergic symptoms were associated. One patient reported fever and painful joints. The time interval between ingestion of the food and the onset of symptoms ranged from 30 to 360 minutes (mean, 125.5 minutes).

SPT carried out in all 38 patients with a history of adverse reactions to seafood was positive for *A simplex* in 33 patients. Among the patients with negative SPT for *A simplex*, 3 reported that they had only ingested raw seafood many years ago. In contrast, patients with positive SPT habitually ingested seafood, either cooked or raw, and in particular raw sardines, which are a common dish in the south of Italy. Positive SPT results were also obtained with the following: shrimp in 2 patients, octopus and crab in 1 patient, cod in 1 patient, and tuna in 1 patient.

RAST was positive in 32 out of 38 patients. No patients had specific IgE against *D pteronyssinus*, *A siro*, *L destructor*, *T putrescentiae*, *A lumbricoides*, or *E granulosus*. RAST values of 1.04 and 0.59 kU_A/L were obtained for shrimp in 2 patients with positive SPT only for *A simplex*. In addition, the patient in whom a positive SPT result was obtained for octopus and crab had RAST values of 1.88 kU_A/L for octopus and 1.40 kU_A/L for crab. The 2 patients with positive SPT for shrimp had RAST values of 2.6 and 8.9 kU_A/L for shrimp, and the patient with a positive SPT for cod had specific IgE concentrations of 6.41 kU_A/L for shrimp, 2.05 kU_A/L for shellfish, 3.07 kU_A/L for tuna, and 4.46 kU_A/L for cod.

Prick-by-prick tests were performed in healthy control subjects and gave negative results in all cases. In the 10 patients sensitized to *A simplex* who met the criteria for prick-by-prick tests, positive results were obtained with live larvae in all cases

and with cooked and frozen larvae in 7 patients (70%). It should be noted that prick-by-prick tests performed in our 10 patients with raw, cooked, and frozen larvae gave results strictly related to preparing or cooking the foods. In fact, 10 patients, 7 who had eaten raw or marinated fish and 3 who had eaten cooked fish, had positive results in prick-by-prick tests with live larvae, 4 of them also had positive results with cooked larvae, and 6 also had positive results with frozen larvae. The 3 subjects who had ingested well-cooked fish had positive results to cooked and live larvae, and 1 of these 3 subjects also had a positive reaction to frozen larvae (Table).

When we performed patch tests with *A simplex* in healthy controls we obtained negative results. In patch tests with the *Anisakis* series, we ruled out the possibility that positive results could be the consequence of an inflammatory skin reaction induced by larval proteases. In fact, after placing larvae on the skin of nonallergic subjects for 48 hours we obtained negative results. Patch tests were positive in 8 patients (80%) for live larvae, in 5 patients (50%) for frozen larvae, and in 1 patient (10%) for cooked larvae (Figure 2). Negative results were obtained for all 3 preparations in 2 subjects. It is important to note that among the 4 patients who reported hand eczema after contact with fresh fish, 2 worked at a fishmonger's and 2 were homemakers.



Figure 2. Results of patch testing with the *Anisakis* series in a positive patient: live (+++), frozen (++), cooked (-).

Discussion

In recent years, IgE-mediated reactions to *A simplex* have been described in Japan [10] and Spain [11-13], where fish consumption is very high. According to Audicana et al [14], approximately 90 g of fish is consumed per person per day in the Basque region of Spain, and 85 g in the rest of Spain. In Japan, the consumption is higher, reaching 239 g per person per day [14]. These factors explain the increase in *Anisakis* sensitization in these regions.

It has been suggested that the ingestion of raw or undercooked fish represents a risk factor for sensitization to

A simplex, and that occupational contact with raw fish is also a risk factor for sensitization, for instance, in fishmongers, fishermen, or workers in fish farms. Some authors have suggested an association between frequent occupational exposure to *Anisakis*-infested fish in people handling fish or fishmeal and *Anisakis*-related allergic disease [15].

In Spain, the prevalence of specific IgE against *A simplex* in patients with urticaria-angioedema is 36%; in healthy donors it is 23% and in children it reaches 56% [16]. In our experience, among all patients seen in our allergy department, 30% of those with specific IgE to food were positive for IgE to *A simplex* (unpublished observations). On the basis of these data, we believe that the incidence of *A simplex* allergy could be underestimated. On the other hand, in the last 9 years reports in the literature of adverse reactions to *A simplex* have risen by 80% [17].

The absence of a clear temporal correlation between the ingestion of parasitized fish and the onset of symptoms represents an element of confusion for the diagnosis of *A simplex* allergy. In our study, the mean delay prior to development of symptoms in the 38 patients examined was 125.5 minutes, while the symptom onset in allergic reactions to food generally occurred 30 to 60 minutes after ingestion. Alonso-Gomez et al [21] also described a delay of 286 minutes before appearance of symptoms in 120 *A simplex*-allergic subjects. This long time interval may be necessary for the release from live larvae of the excretory-secretory (ES) proteins, including Anis s 1 (the major allergen), responsible for allergic reactions.

From a functional and structural point of view, *A simplex* has 3 antigens that are responsible for an immune response in the parasitized host: (1) somatic antigens with a molecular mass of between 13 and 150 kDa [18]; (2) ES antigens, which are proteolytic and hyaluronidase enzymes that allow the penetration of *A simplex* into gastric mucosa [18,19]; and (3) superficial antigens, which are present in the parasite cuticle and probably involved in the chronic inflammation [20]. The allergenic molecules identified in *A simplex* at the L3 stage are Anis s 1, which represents the major allergen (24 kDa) [22] and belongs to the ES antigens [23]; Anis s 2 (also named paramyosin), a somatic antigen [24]; Anis s 3, which corresponds to a tropomyosin [25]; and Anis s 4, which is an allergen with a low molecular mass (9 kDa) of somatic origin, characterized by high thermostability and pepsin-digestion stability [26].

In the literature, data on the allergenicity of cooked and frozen larvae are contradictory, and many studies have suggested that only the presence of live worms is able to provoke hypersensitive reactions in humans [3]. Alonso-Gomez et al [21] demonstrated, in 22 patients with gastrointestinal and allergic symptoms provoked by *A simplex*, that the ingestion of frozen larvae was tolerated. In fact, oral challenge tests performed using *A simplex* larvae stored at -20°C for 48 hours were negative in all subjects.

Sastre et al [27] obtained negative results after oral challenge with lyophilized larvae in 12 *A simplex*-sensitized patients with a history of anaphylaxis induced by raw or marinated fish parasitized by *A simplex*. Moreover, it should be noted that since lyophilization of larvae can induce a loss of

allergenicity of larval proteins, this method does not reproduce natural exposure to allergens. At the same time, it is important to highlight that *A simplex* larvae can survive in vinegar for up to 51 days [28]. Patients with positive results in prick-by-prick tests with cooked larvae also had positive reactions to live larvae. These data are suggestive of dual sensitization to different allergens: live larvae could induce sensitization by penetrating the gastric mucosa and releasing ES antigens, while the probable allergen responsible for adverse reactions to cooked larvae would be Anis s 4. This allergen, has a high resistance to heat and pepsin digestion [22,26].

To explain the IgE-mediated response to cooked larvae, we can also hypothesize another mechanism in which, after penetration of the mucosa, the larvae die and spontaneously release antigenic material characterized by somatic antigens, inducing patient sensitization and, hence, an immediate reaction on subsequent contact with cooked fish containing dead larvae [29]. We can therefore hypothesize that subjects with positive responses to cooked larvae could previously have come into contact with live larvae by ingesting raw or undercooked fish, but without developing symptoms. In fact, we found a dual positivity in these patients. In 4 patients who reported eczema after contact with raw fish, we obtained highly positive reactions to live larvae after patch testing with the experimental *Anisakis* series, while cooked larvae gave positive results in only 1 of them. Two of the 4 also had positive reactions to frozen larvae. There was an improvement of the symptoms during rest periods in 2 patients with occupational exposure to *A simplex*, followed by reappearance of the symptoms when they returned to work. In the remaining 6 patients who presented IgE-mediated symptoms, we found positive patch-test results for live larvae in 4 and positive reactions to frozen larvae in 3, while none of them had positive reactions to cooked larvae.

IgE-mediated sensitization is not influenced by cooking or freezing the fish and is elicited by antigens from the parasite that are unrelated to fish proteins [30]. In fact, even though it is possible to kill the parasite by cooking and freezing, the allergenic capacity is not necessarily destroyed [31]. Our data indicate that *A simplex* can elicit not only immediate allergic reactions but also delayed-type allergic reactions in subjects who have repeated direct contact with parasitized fish. In our study, live larvae were most frequently responsible (in 80% of cases) for cell-mediated reactions. Analysis of the symptoms reported by the patients revealed that only 1 in 10 patients suffered simultaneously from urticaria and gastrointestinal symptoms. These aspects are underlined by Audicana et al [14], who found that *A simplex* was the main factor responsible for urticaria and angioedema following fish consumption in adults, and that the digestive complaints were the second most commonly reported symptom. In our study, allergic reactions were more frequent than the clinical manifestation referred to by Daschner et al [32,33] as gastro-allergic anisakiasis, characterized by simultaneous gastric symptoms and allergic hypersensitivity (urticaria, angioedema, or anaphylaxis) symptoms. This syndrome is provoked by fish parasitized by *A simplex* and its onset is only induced by live larvae, which are able to penetrate the gastric mucosa by releasing proteases. We can hypothesize that in patients with exclusively allergic

reactions the contact between the nematode and the gastric or intestinal mucosa required to induce gastric symptoms is very short, because in the literature 9 subjects reported urticaria-angioedema syndrome associated with itching and 3 had anaphylactic shock [32]. In 1990, Kasuya et al [10] reported allergic symptoms without the simultaneous presence of anisakiasis symptoms. Even though Lopez-Serrano et al [34] have suggested that allergic reactions to *A simplex* alone are rare, reports from other authors indicate frequent associations with urticaria and angioedema [2], anaphylaxis [35], and digestive symptoms [32]. One of those studies emphasized that anaphylaxis can develop either after infection or from mere exposure to the antigen and that the allergen could be resistant to cooking and freezing the fish, thus allowing a higher frequency of adverse reactions in countries where the consumption of fish is high [35]. The observation of cutaneous lesions suggests that a more diligent evaluation of chronic eczema defined as idiopathic is needed in patients living in coastal areas where fish are very frequently handled.

In conclusion, we believe that further studies must be performed to identify the proteins responsible for delayed allergic reactions provoked by live, cooked, and frozen larvae so that an *Anisakis* series can be prepared for patch testing using standardized haptens, which are currently unavailable. In addition, in order to avoid sensitization to *A simplex* it is important to observe the European ruling that suggests freezing fish to be consumed raw at -20°C for 48 hours, even if this recommendation is not completely valid because it does not lead to total inactivation of allergenic proteins. Since the testing carried out in fish markets is usually done on random samples, it does not guarantee a lack of contamination in all fish. Finally, as a prophylactic measure, it is of key importance to purchase only fresh fish and eviscerate it rapidly to prevent the larvae from passing through the celomic cavity to the edible part (the muscle fibers).

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