

Short communication

A double-blind, placebo-controlled oral challenge study with lyophilized larvae and antigen of the fish parasite, *Anisakis simplex*

Background: The third-stage larvae of *Anisakis simplex* may be a hidden source of allergens in fish. The objective was to determine whether the ingestion of lyophilized *A. simplex* larvae, or antigen, induces clinical symptoms in a group of *A. simplex*-sensitized patients.

Methods: Double-blind, placebo-controlled oral challenges were conducted in 11 individuals who had experienced allergic reactions after eating fish. Another patient had chronic urticaria unrelated to the ingestion of fish. All patients had positive skin tests and specific IgE determinations for *A. simplex* and negative skin tests to a battery of fish species. Conjunctival tests with *A. simplex* extracts were conducted in all patients and in five controls. The 12 patients received capsules containing either lactose or one, five, or 25 lyophilized larvae of *A. simplex* at 2-h intervals in a double-blind fashion. The highest single dose was 100 larvae. ECP and tryptase levels in serum were measured before and after the last oral challenge. Lyophilized antigen was also given to five patients.

Results: None of the 12 patients experienced a positive reaction after the ingestion of the placebo, the lyophilized larvae, or the antigen. Tryptase and ECP levels before and after challenges did not change significantly. Conjunctival provocation tests were positive in 11 out of the 12 patients and in none of the controls.

Conclusions: The ingestion of 100 lyophilized *A. simplex* larvae, or its equivalent in antigen, does not induce clinical symptoms in individuals with a clinical history and laboratory findings of hypersensitivity to *A. simplex*. The data suggest that only the ingestion of live larvae may be capable of inducing allergic manifestations.

J. Sastre, M. Lluch-Bernal, S. Quirce

Servicio de Alergia, Fundación Jiménez Díaz, Universidad Autónoma de Madrid, Madrid

I. Arrieta, C. Lahoz, A. Del Amo

Servicio de Inmunología, Fundación Jiménez Díaz, Universidad Autónoma de Madrid, Madrid

E. Fernández-Caldas, F. Marañón

C.B.F. LETI, SA, Research Laboratories, Tres Cantos, Madrid, Spain

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Joaquín Sastre, MD, PhD
Servicio de Alergia
Fundación Jiménez Díaz
Avenida Reyes Católicos 2
28040 Madrid
Spain

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Anisakis simplex is an anisakid nematode (phylum: Nematoda, class: Rhabditea, order: Ascaridida, superfamily: Acaridoidea, family: Anisakidae, subfamily Raphidascaridinae) that commonly parasitizes fish (1–3). The third-stage (L3) larva has been recognized as a source of hidden allergens in fish (1, 4, 5). The ingestion of *A. simplex* can produce clinical disorder in man (6); i.e., anisakiasis, which is a parasitic disease (acute or chronic) caused by the accidental ingestion of live *A. simplex* larvae in raw or improperly prepared fish and squid dishes, and hypersensitivity reactions after the ingestion of supposedly parasitized fish. These allergic reactions are thought to be mediated by specific IgE to *A. simplex* allergens.

Several communications from Japan and Spain have reported that patients who develop allergic symptoms (urticaria/angioedema and anaphylaxis) after the ingestion of parasitized fish are frequently sensitized to allergens of this nematode (1, 4, 5, 7). The diagnosis of clinical hypersensitivity to *A. simplex* is hampered by

the difficulty in visualizing and identifying the parasites within the fish and relating the clinical symptoms of the patient to the ingestion of *A. simplex*. Several clinical manifestations have been associated with hypersensitivity to *A. simplex*. Occupational conjunctivitis (8) and asthma (9), supposedly induced by *A. simplex*, have been reported. The allergenicity and cross-reactivity of other fish parasites has also been documented (10).

In spite of recent advances in the study of *A. simplex*-induced allergic reactions, the underlying mechanisms remain to be established. Recent evidence suggests that only the ingestion of live larvae, which sometimes attach themselves to the human gastric mucosa, are responsible for these reactions (11–13).

Herein we describe the results of a prospective double-blind, placebo-controlled oral provocation study with lyophilized (L3) larvae of *A. simplex* and conjunctival provocation tests in a group of patients with a clinical history suggestive of reactions to *A. simplex*, and both skin tests and *in vitro* data confirming

Table 1. Clinical characteristics of patient population and controls

Patient no.	Age (years)	Sex	Atopy	Positive skin test	Episodes	CT	DBPCOT
1	19	F	Yes	Pollens	3	Negative	Negative
2	53	F	No	—	6	Positive	Negative
3	52	F	Yes	Pollens	2	Positive	Negative
4	61	M	No	—	1	Positive	Negative
5	52	F	No	—	1	Positive	Negative
6	63	F	No	—	1	Positive	Negative
7	36	F	No	—	3	Positive	Negative
8	45	M	Yes	Pollens, mites	1	Positive	Negative
9	26	F	No	—	1	Positive	Negative
10	28	F	Yes	Pollen	1	Positive	Negative
11	34	M	No	—	1	Positive	Negative
12	26	M	Yes	Pollens	0	Positive	Negative

Episodes: number of previous allergic episodes related to fish ingestion.

CT: result of conjunctival challenge test with *A. simplex*.

DBPCOT: result of double-blind, placebo-controlled oral challenge test with lyophilized *A. simplex*.

sensitization to this fish parasite. The protocol was revised and approved by the Institutional Review Board of the Fundación Jiménez Díaz Hospital and the Drug Evaluation Committee of the Spanish Department of Health (document no. 97/318).

Material and methods

Patient population

The patient population consisted of 11 individuals who had experienced several allergic reactions (urticaria/angioedema, abdominal pain, or anaphylaxis) after eating marinated fish. Another patient (no. 12) had chronic urticaria unrelated to the ingestion of fish, but positive skin test and specific IgE determinations to *A. simplex* antigens. The demographic characteristics are shown in Table 1. All 12 patients had positive skin tests to various concentrations of *A. simplex* extract (C.B.F. LETI, SA, Madrid, Spain) and a positive specific IgE determination by CAP (Pharmacia & Upjohn, Sweden). Patients were also skin tested with a battery of common aeroallergen and food extracts, including nine fish species, squid, clam, and shrimp. One hundred consecutive atopic individuals were skin tested with a 1 mg/ml extract of *A. simplex*.

Preparation of the allergenic materials

Two types of allergenic materials were prepared, consisting of lyophilized larvae for oral provocation challenges and lyophilized *A. simplex* extract for skin and conjunctival tests and also for oral challenges.

Live larvae of *A. simplex* were collected from *Micromessistius poutassou* (bacaladilla, Couch's whitening) and *Gadus merluccius* (merluza, hake) with forceps and identified by expert parasitologists. Extracts were prepared by methods previously described (10). An amount of 20 g of larvae was frozen at -80°C for 5 days and lyophilized for 3 days. Approximately half of

the lyophilized larvae was crushed and extracted 1:50 w/v in ammonium bicarbonate, 0.125 mol/l, pH 8.2, for 16 h at 4°C ; the mixture was then centrifuged, the pellet discarded, and the remaining solution passed through a 0.22- μm filter, dialyzed in 3.5-kDa membranes, and lyophilized. In this extract, the protein concentration, as measured by the Lowry method, was 54.5% of the dry weight (540 $\mu\text{g}/\text{mg}$ of lyophilized material). The weight of one live larva was estimated at 3.8 mg and that of a freeze-dried larva at 0.9 mg. The yield of the extract was 19%.

Skin prick tests

Three concentrations (0.1, 1, and 4 mg/ml) of the *A. simplex* extract were tested in duplicate. Histamine (10 mg/ml) and glycerinated saline were used as controls. Standardized aeroallergens, food extracts (C.B.F. LETI, SA, Spain), and raw fish (tested prick by prick) were used. A test was considered positive when the wheal was greater than 3 mm in the absence of a positive reaction to the negative control.

Conjunctival provocation test

A conjunctival provocation test with the lyophilized extract was performed and interpreted by the method of Möller et al. (14). Briefly, one vial containing 1 mg of lyophilized *A. simplex* extract was reconstituted with sterile saline solution. Three dilutions were used containing 0.1, 1, and 4 mg/ml. One drop of increasing concentrations was applied in the conjunctival sac. A reaction was considered positive when the patient developed erythema and pruritus of the conjunctiva. All the reactions were evaluated by the same physician 20 min and 3 and 6 h after the test was conducted. A control saline solution was used in the other eye.

Five patients with chronic idiopathic urticaria

unrelated to the ingestion of fish but with positive prick tests and CAP to *A. simplex* were used as controls in the conjunctival challenges.

CAP, ECP, and tryptase determinations

Serum ECP and tryptase levels (UniCAP Pharmacia & Upjohn, Sweden) were measured according to the manufacturer's instructions before and 1 h after the last oral challenge. Specific IgE by the CAP method to *Ascaris lumbricoides* and *Taenia equinococcus* were also measured. Levels below 11.3 and 13.5 µg/l of ECP and tryptase, respectively, may be considered normal according to the instructions of the manufacturer.

Double-blind, placebo-controlled oral challenges

Patients received placebo or lyophilized *A. simplex* larvae in a double-blind fashion. Gelatin capsules were prepared containing either lactose or 1, 5, 10, and 25 lyophilized larvae of *A. simplex*. Vials containing the capsules were labeled in a blinded manner, and the physicians conducting the challenges were not aware of the content of the capsules. Challenges were performed on different days, the patient receiving at least two capsules per day, up to a last dose of 100 larvae. One challenge contained placebo and another one the larvae. Patients were closely followed in the hospital by medical personnel for 8 h and were instructed to contact the physician on call if any symptoms developed. The patients were seen again after 24 h.

On a separate day, five patients also received 20 or 40 mg of lyophilized *A. simplex* extract (equivalent to approximately 105 or 210 larvae, respectively), reconstituted in 1 ml of distilled water, in a double-blind fashion.

Results

Skin tests

All the patients had a positive skin test reaction to the

A. simplex extracts. The largest reaction was obtained with 4 mg/mg, although all the patients reacted to the three concentrations tested. None of the patients had a positive reaction to the fish extracts tested. Four patients were allergic to grass pollen and one patient to grass pollen and mites. One hundred consecutive atopic patients without a history suggestive of clinical sensitivity to *A. simplex* were also tested with a 1 mg/ml *A. simplex* extract; nine (9%) had a positive reaction.

Conjunctival provocation tests

In conjunctival provocation tests, 10 patients had an immediate positive reaction; one had a dual reaction (patient 3). Patient 12 experienced only a late-phase reaction after 3 h. The five control subjects, with chronic idiopathic urticaria and positive skin tests to *A. simplex*, had negative conjunctival tests.

Oral provocation tests

None of the 12 patients experienced a positive reaction (immediate or late) after ingesting the placebo, the capsules, or the aqueous extract containing *A. simplex*. Twelve patients received a final dose of 100 larvae in capsules. Five of these patients were also challenged with a liquid extract; three received 20 mg of the extract (patients 9, 11, and 4) and two patients 40 mg (equivalent to 210 larvae; patients 8 and 10).

CAP, ECP, and tryptase results

IgE and CAP results for *A. simplex*, *Ascaris lumbricoides*, and *Taenia equinococcus* are summarized in Table 2. Mean ECP and tryptase levels before the test were 10.33 ± 5.73 µg/l and 6.15 ± 2.46 µg/l, respectively. One hour after the last oral challenge, mean ECP and tryptase levels were 11.21 ± 4.07 µg/l ($P=0.58$) and 5.71 ± 2.28 µg/l ($P=0.38$). No significant differences were observed in ECP and tryptase levels before and after the challenges.

Discussion

In 1990, Kasuya et al. (1) published the first cases of mackerel (*Trachurus trachurus*)-induced urticaria related to the ingestion of *A. simplex*-parasitized fish. The authors concluded that their hypothesis could be tested more conclusively only by provocation studies. Herein, we report a double-blind, placebo-controlled oral challenge study with lyophilized *A. simplex* larvae and liquid extract. The results suggest that the ingestion of up to 100 lyophilized larvae of *A. simplex*, or its equivalent in lyophilized antigenic material, did not induce clinical symptoms in sensitive individuals with a very suggestive clinical history and laboratory findings of sensitivity to *A. simplex*.

As part of this study, we evaluated the prevalence of

Table 2. Total and specific IgE results in patients

Patient no.	Total IgE in kU/l	CAP* <i>A. simplex</i>	CAP* <i>As. lumbricoides</i>	CAP* <i>T. equinococcus</i>
1	153	3	0	0
2	164	5	0	0
3	830	6	3	1
4	1031	6	0	0
5	754	6	3	0
6	2222	6	2	1
7	36	3	0	0
8	637	4	2	0
9	753	6	3	2
10	45	3	0	0
11	960	6	0	0
12	124	4	0	0

CAP*: results in CAP classes.

positive skin test reactions to *A. simplex* in 100 consecutive patients evaluated for upper and/or lower airway allergic respiratory complaints; nine of these individuals had a positive skin test, although none of them reported a clinical history of reactions after fish ingestion. These results are similar to those previously described, considering that the patient population evaluated did not complain of any fish-related allergies (6). In Japan, specific IgE to *A. simplex* has been detected in 33% of patients with atopic dermatitis, 75% of patients with fish-induced urticaria, and 10% of controls (6). In Spain, the prevalence of specific IgE to *A. simplex* antigens in patients with urticaria/angioedema is 36%; in blood donors, 23%; and in children with high total IgE levels, 56% (6).

In an attempt to establish clinical sensitivity, conjunctival provocation tests were conducted in the 17 individuals included in this study. All these individuals had a positive skin test and positive specific IgE determination to *A. simplex*. Eleven of these 17 individuals (64.7%) had a positive conjunctival test. It is noteworthy that 10 of the 11 individuals (90.9%) with a history of hypersensitivity reactions after fish ingestion had a positive conjunctival challenge test. These results suggest that the conjunctival test is a useful diagnostic tool to identify patients with a clinical history suggestive of *A. simplex* hypersensitivity. Recently, two cases of occupational conjunctivitis and asthma have been diagnosed by the results of organ-specific challenges (8, 9).

Oral challenges were conducted with two kinds of allergenic material, lyophilized larvae and liquid extract; none of the patients tested had a positive oral challenge. Several explanations may account for these findings. The first possibility is that the lyophilized larvae are not the ideal material for oral challenges because of a potential loss of allergenicity during the lyophilization process. However, it was demonstrated by skin prick tests, conjunctival challenges, and specific IgE determinations that the larvae retained their allergenicity after lyophilization, since the material used in these tests originated from lyophilized larvae. Other studies have shown that the ingestion of lyophilized food induces positive challenge results in sensitized patients (15).

Second, the amount of larvae ingested by the study population was not enough to produce symptoms; more was needed to elicit an adverse reaction. The amount of larvae present in fish varies greatly; it depends on the species, the fishing area, and the time of the year the fish is caught. It also depends on the part of the fish body ingested. The parasites most commonly form cysts in the body cavity, gut, and organs, especially the liver. Larvae are less likely to be found in muscle tissue. For several fish species caught off the northern Spanish coast, it has been reported that, although the number varies greatly, a parasitized fish of normal size may contain 1–20 larvae (13). Commonly, fish are carefully

cleaned before ingestion and inspected for parasitization, especially since, in recent years, consumers have become more aware of this problem. The consumption of raw, uncooked, or marinated fish may increase the risk of ingesting live larvae (2).

Third, it is possible that the allergen present in the larvae may be destroyed in the stomach of the patient. *A. simplex* larvae have been shown to be very stable under various conditions and contain heat-stable, acid-resistant proteins (2, 14). Patients were also challenged with lyophilized extract. Five patients ingested 20 or 40 mg of lyophilized antigen. Again, none of these patients showed any clinical symptoms.

Fourth, clinical allergic manifestations, such as anaphylaxis, urticaria, or angioedema in *A. simplex*-sensitized patients occurs, in most cases, with the parasitization of the gastric mucosa of the patients by the nematode, as has been suggested in three reports (11–13). Our results corroborate these findings, suggesting that only live larvae may induce clinical symptoms in sensitized patients, as suggested by Alonso et al. (11). These authors administered one, four, and six frozen larvae to five patients with “gastroallergic anisakiasis” in whom a live larva had been previously removed from the stomach; none of them reacted on open challenge to the ingestion of a total of 11 frozen larvae.

It seems that the allergen or allergens responsible for clinical manifestations are produced by live larvae as secretory antigens, and thus may not be present in sufficient quantities in dead specimens. A collagenolytic proteinase produced in the esophagus of this nematode is responsible for tissue invasion and successful colonization of the host (2), and could be responsible for the clinical manifestations seen in patients after parasitization.

Despite the results obtained in this study, we cannot conclude that the ingestion of fish parasitized by *A. simplex*, or other parasites (17), is safe for sensitized individuals. The reason that individuals become sensitized to *A. simplex* antigens also remains to be established, although the ingestion of parasitized fish seems to be the most plausible cause. Cross-reactivity with other nematodes or insects may also account for some cases (18).

Based on this and previous studies, *A. simplex*-sensitized patients should avoid the ingestion of raw, undercooked, and/or marinated or pickled seafood. The consumption of fish that have gone through the process of candling, heading, gutting, trimming, and freezing immediately after capture may contribute to the control of the risks posed by *A. simplex* (19). Effective ways of killing the parasite are freezing and heat inactivation (19). Our patient population has been advised to avoid raw, undercooked, or marinated dishes, but allowed to continue to eat frozen fish dishes; so far no relapses have occurred.

We acknowledge that challenging the patients with lyophilized larvae or extract may not be the most natural way of repeating a natural exposure, but in the context

of the ethical problem posed by the challenges with live larvae, this is the nearest we can get to natural exposure. Parasitization of the gastric mucosa by live larvae of *A. simplex* seems to be needed to trigger the allergic manifestation seen in *A. simplex*-hypersensitive patients.

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