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Original Article

Double-blind, placebo-controlled oral challenge study with lyophilised *Anisakis simplex* larvae and antigen

Multiple allergic hypersensitivity reactions to the fish parasite *Anisakis simplex* have been documented and reported since the 1990s. The present study reports the results of a double-blind challenge study with oral administration of lyophilised *Anisakis simplex* larvae or placebo. Eleven patients with a history of anaphylactic reactions after fish intake and a further one with chronic urticaria unrelated to fish ingestion were enrolled in the study. All patients had positive skin and CAP-System tests to *A. simplex*. All of them underwent testing with a panel of airborne allergens and fish and conjunctival challenge testing with an *A. simplex* extract (1 mg/ml). Gelatine capsules containing either lactose (placebo) or 1, 5, 10 or 25 lyophilised *A. simplex* larvae were prepared. The subjects receiving, according to a double blind, 2-hour-interval protocol, one capsule containing either placebo or larvae. Ten patients, and none of five controls, evidenced positive conjunctival tests. None of the twelve patients evidenced adverse reactions of any type after the oral challenge test. These results suggest that the ingestion of lyophilised *A. simplex* larvae does not cause clinical symptoms in sensitised patients, even when the patients had highly suggestive histories and analytical evidence of hypersensitivity. Based on our findings, we cannot conclude that the intake of fish parasitised by *Anisakis simplex* represents a safe alimentary habit. Such intake may induce clinical manifestations in sensitised patients. Nevertheless, our data suggest that lyophilised or non-viable larvae may not be a sufficient stimulus for prompting allergic symptoms.

Key words: *Anisakis simplex*. Antigen. Lyophilised larvae.

Estudio de provocación oral doble ciego controlada con placebo con larvas y antígeno de *Anisakis simplex* liofilizados

Desde los años 90 se han descrito numerosos casos de reacciones de hipersensibilidad producidas por *Anisakis simplex*, un parásito del pescado. En este trabajo se describen los resultados de un estudio con provocaciones doble ciego frente a placebo con larvas liofilizadas de *Anisakis simplex*. Se han estudiado un total de 11 pacientes con historia de reacciones anafilácticas tras la ingestión de pescado y otro paciente con urticaria crónica sin relación con la ingesta de pescado. Todos los pacientes tenían pruebas cutáneas y CAP positivos a *A. simplex*. Se realizaron pruebas cutáneas con una batería de neumoaergenos y pescados, así como una prueba de

provocación conjuntival con un extracto de *A. simplex* (1 mg/ml). Se prepararon cápsulas de gelatina que contenían lactosa o bien 1, 5, 10 ó 25 larvas liofilizadas de *A. simplex*. Cada día, y con intervalos de dos horas, los pacientes recibían en doble ciego cápsulas de placebo o de larvas. Diez pacientes, pero ninguno de los cinco controles, tuvieron prueba conjuntival positiva con *A. simplex*. Ninguno de los doce pacientes presentó reacción alguna tras la provocación oral. Estos resultados sugieren que la ingestión de larvas liofilizadas no induce síntomas clínicos en pacientes sensibilizados, incluso con historia y datos analíticos altamente sugestivos de hipersensibilidad. A pesar de los resultados obtenidos en este estudio, no podemos concluir que la ingestión de pescado parasitado por *Anisakis simplex* sea segura, ni que la ingestión de éste no produzca manifestaciones clínicas en pacientes sensibilizados. Sin embargo, nuestros resultados sugieren que las larvas liofilizadas o no viables pueden no ser estímulo suficiente para desencadenar síntomas alérgicos.

Palabras clave: *Anisakis simplex*. Antígeno. Larvas liofilizadas.

INTRODUCTION

Since the first report by Kasuya et al. in 1990, a considerable number of hypersensitivity reactions induced by this fish parasite have been reported¹⁻⁵. The ingestion of the

parasite by humans may induce infestation –anisakiasis– and hypersensitivity reactions in subjects sensitised to it⁶. Diagnosis of the hypersensitivity reactions has been hampered by the difficulty in knowing whether the suspect fish was actually infested by this parasite and whether the parasite was alive or dead at the time of ingestion^{1-5,7}. Episodes of occupational conjunctivitis and asthma have also been recently reported in association to sensitization to this parasite^{8,9}. Cross-reactivity to other parasites has also been reported¹⁰. Some publications suggest that only the ingestion of the live parasite is able to induce hypersensitivity phenomena¹¹⁻¹³. We here present a summary of the results of a double blind, placebo-controlled oral challenge study using lyophilised larvae and aqueous extract of *Anisakis simplex*. A more detailed account of the study has been published in Allergy 2000; 55:560-564.

MATERIALS AND METHODS

Patients and controls

The study group comprised eleven patients with a highly suggestive history of anaphylactic reactions after ingestion of fish (vinegar-marinated fish in many cases) and with positive skin tests and specific IgE (CAP) to *Anisakis simplex*, and one patient with chronic urticaria bearing no relation to fish ingestion, but also with positive skin tests

Tabla I. Clinical characteristics of the patients and results of the conjunctival and oral challenge tests

Patient No.	Age	Gender	Atopy	No. of episodes	Conjunctival challenge	Oral challenge
1	19	F	Yes / pollens	3	Negative	negativa
2	53	F	No -	6	Positive	negative
3	52	F	No / 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 / pollens	1	Positive	negative
11	34	M	No -	1	Positive	negative
12	26	M	Yes / pollens	0	Positive	negative

No. of episodes: number of previous allergic episodes related to fish ingestion.

Conjunctival challenge: results of the conjunctival challenge with *A. simplex* extract.

Oral challenge: results of the double blind placebo controlled challenge with lyophilised *A. simplex* larvae.

Table II. Results of the total serum and specific (CAP) IgE measurements in the patients

Patient No.	Total serum IgE	<i>Anisakis simplex</i>	<i>Ascaris lumbricoides</i>	<i>Taenia echinococcus</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

and specific IgE (CAP) to *Anisakis simplex*. Table I summarises some clinical characteristics of these patients.

Two control groups were used in the study. The first one, which was to serve as a control for the conjunctival challenge tests, comprised five patients with chronic urticaria not related to fish ingestion but with positive skin tests and specific IgE to *A. simplex*. The second one comprised one hundred consecutive patients seen at our outpatient clinic for study of respiratory allergy. This group served as the control for the skin tests with *A. simplex* extract.

Extracts and tests

Airborne allergens: mites, cockroaches, pollens, moulds, epithelia (CBF Leti, Madrid, and ALK-Abelló, Madrid, Spain).

Fish: commercial extracts (CBF Leti) and prick-by-prick (12 species).

Anisakis simplex: live larvae were lyophilised and extracted 1:50 (w/v) in ammonium bicarbonate 0.125 M, pH 8.2; the extract was centrifuged, filtered and dialysed through a membrane with a 3.5 kD cut-off point. The final protein content was 54% of the dry weight, and the yield was 19% of the starting value¹⁰.

For the prick tests and for the conjunctival challenge with the *Anisakis simplex* extract, 0.1, 1.0 and 4.0 mg/ml concentrations were used. For the oral challenge tests with aqueous oral extract, the concentrations used were 20 or 40 mg/ml, equivalent to 105 and 210 larvae, respectively.

Gelatine capsules were prepared for the double blind oral challenge tests containing lactose (placebo) or 1, 5, 10 or 25 lyophilised larvae.

Conjunctival challenge tests: different concentrations of the aqueous extract (0.1 – 1.0 – 4.0 mg/ml) were instilled in one conjunctival sac, the diluent alone being instilled into the contralateral one¹⁴. The test results were assessed after 20 min and again after 4-6 hours.

Oral challenge tests: these tests were carried out in the double blind placebo-controlled modality. Two capsules a day were administered, with intercalated placebo, for a maximum possible dose of 100 larvae at a single administration. In the case of the aqueous extract, 1 ml of the extract (20 or 40 mg/ml) or of the placebo solution was given. The clinical symptoms were monitored up to 48 hours after the challenge.

For the ECP and tryptase determinations (Pharmacia, Uppsala, Sweden), blood samples were collected according to the manufacturer's instructions at baseline and again either one hour after the occurrence of any clinically manifest reaction or after concluding the last challenge.

In the patients in the study group, the total serum IgE levels were measured, as well as the specific IgE (CAP System) antibodies to *Ascaris lumbricoides* and *Taenia echinococcus*.

RESULTS

Skin tests

The skin tests with *A. simplex* extract were positive in 9% of the control patients selected among those attending our outpatient clinic because of supposed respi-

Tabla III. Eosinophil cationic protein and tryptase levels before and after the oral challenge tests in the patients

Patient No	ECP (µg/l)		Tryptase (µg/l)	
	Baseline	Final	Baseline	Final
1	2	3.7	1.79	3.72
2	16.5	7.2	5.4	5.1
3	15.1	11.3	9	8.7
4	12.7	12	6.1	7
5	7.3	13.3	10.4	10
6	12.4	14.4	8	5.5
7	6.8	12.3	4	2.9
8	19.5	19.2	4.1	4
9	2.4	7.9	6.9	8.1
10	14.5	10.3	4	4.1
11	10.4	8.5	8.1	3.5
12	4.4	14.5	6.1	6

ratory allergy. None of the patients in the study group (history highly suggestive for *A. simplex* sensitization) or of the controls evidenced positive skin tests with fish (Table I).

Conjunctival challenge tests

The conjunctival challenge with *A. simplex* extract was negative in all five control patients with chronic urticaria and with positive skin tests and CAP for *A. simplex*.

In the study group, 10/11 patients (90.9%) with a highly suggestive history had positive conjunctival challenge tests, in one case with a dual reaction (Patient No. 3) and in a further one with an exclusively delayed one (Patient No. 12) (Table I).

Oral challenge tests

The oral challenge tests with capsules (12 patients) and with the aqueous extract (5 patients) were negative both with the placebo and with the active preparation in all patients in the study group (Table I).

Other results

The results of the total IgE quantification and of the CAP-System determinations for *A. lumbricoides* and *T. echinococcus* are summarised in Table II. The results of the serum ECP and tryptase determinations are presented in Table III.

DISCUSSION

The results of the present study suggest that the ingestion of lyophilised *Anisakis simplex* larvae do not indu-

ce clinical symptoms in sensitised patients, even in those whose clinical histories and laboratory results are highly suggestive of sensitization to the parasite. However, the oral challenge tests with lyophilised foodstuffs have been positive in other studies on food allergy¹⁵.

Among the patients attending our outpatient clinic because of suspected respiratory allergic disease we detected 9% positive skin tests with an *Anisakis simplex* extract. In other studies performed in Japan, the positivity proportions were 10% among control subjects, 33% among patients with atopic dermatitis, and 75% among patients with chronic urticaria in relation to fish ingestion. In Spain, the overall prevalence is 23% among otherwise healthy blood donors, 36% among patients with urticaria and 56% among children with high total serum IgE levels⁶.

The results of the conjunctival challenge test, which was positive in 10 out of 11 patients (90.9%) with clinical manifestations highly suggestive of sensitization and negative in five controls who however had positive specific *Anisakis simplex* IgE, would appear to suggest that this test might have diagnostic value; however, the numbers of patients studied are still too small for drawing definitive conclusions.

It might be argued that the amount of larvae ingested during the tests by the study patients had been insufficient for triggering allergic manifestations. However, the fish from our own coastal regions we have analysed have an average infestation level of 1 to 20 larvae¹³, while our patients ingested up to 100 larvae in their last challenges. A low heat- and acid-stability of the extract used may also be argued; however, we have previously demonstrated that *Anisakis simplex* extracts contain proteins that are heat-stable and acid-stable^{2,14}.

The results of the present study also appear to corroborate those of other studies, performed with smaller numbers of patients, which suggest that only the live viable larva infesting the gastric mucosa is able to induce clinical symptoms in sensitised patients¹¹⁻¹³. Thus, it would seem that the allergen or allergens responsible for the clinical manifestations might be present in only small quantities in the dead parasites, and that some collagenolytic enzyme produced in the parasite's oesophagus might be responsible not only for the attachment of the parasite to the gastric mucosa but also for the allergic manifestations in sensitised individuals.

In spite of the results achieved in the present study, we cannot conclude that the ingestion of fish parasitised by *Anisakis simplex* or by other parasites^{16,17} may represent a safe practise, nor that the ingestion of such fish may not induce clinical manifestations in sensitised patients. However, it would appear that lyophilised larvae do not represent a sufficient stimulus for triggering allergic symptoms. The one recommendation that can be derived from the results of the present study is that sensitised individuals should not consume raw or undercooked fish, and that previous deep-freezing might perhaps prevent the development of hypersensitivity phenomena.

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