

Characterization of allergens secreted by *Anisakis simplex* parasite: clinical relevance in comparison with somatic allergens

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Summary

Background Diagnostic methods for the study of allergic reactions to *Anisakis simplex* (*A.s.*) based on whole-body extracts of the larva are clearly insufficient.

Objectives To study the allergenicity of the proteins secreted by the parasite. Comparison with somatic antigens and determination of their clinical importance in allergic patients were also addressed.

Methods An excretory/secretory (E/S) extract was produced by culturing third-stage *A.s.* larvae. It was used to perform immediate skin tests and to determine specific IgE in 10 patients diagnosed with allergy to *A.s.* Both tests were compared with the results obtained with the whole-body extract (somatic (S)). The molecular weight (MW) of their allergens was determined by immunoblotting, and a single-blind placebo-controlled oral challenge with E/S proteins was performed. Finally, allergens' resistance to gastric pepsin and acid pH was explored.

Results *A.s.* larvae secreted allergens more potent than those present in the S extract. The skin prick test wheal area produced by E/S molecules and the absorbance obtained in the determination of specific IgE with these allergens (ELISA) were 5.8 times bigger than those obtained with S extract. MW allergens of 72 and 56 kDa in E/S extracts and those of 56, 48 and 43 kDa in S extract were recognized by more than 50% of the patients. Partial cross-reactivity between them was revealed by immunoblotting inhibition studies. Oral challenge with E/S extract (up to 479 µg) was negative in all the patients. Treatment of E/S proteins with gastric pepsin inhibited the binding of the E/S allergens for specific IgE. The acid pH did not affect the overall binding of IgE to E/S extract. It decreased by 15.23% and 19.96% at pH 4 and 2, but the difference was not statistically significant.

Conclusion *A.s.* secretes allergens more potent than somatic antigens and should be used in the diagnostic procedures. These allergens are inactivated by the pepsin, which supports the theory that live larva is necessary to induce an allergic reaction in most of the patients.

Keywords allergens, *Anisakis simplex*, fish, food allergy, parasite

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Introduction

Anisakis simplex (*A.s.*), a nematode of the Anisakidae family, can infest humans when raw or undercooked marine products are eaten and may cause Anisakiasis or Anisakidosis [1–3]. The penetration of the cephalic portion of the parasite through the digestive mucosa and the consequent release of toxic substances cause local inflammation. This disease is avoided if the fish is previously frozen for 48 h at -20°C .

In 1990, the first recognized case of allergic reaction to the *A.s.* protein was described in Holland [4]. The allergic mechanism requires previous contact with the parasite (sensitization) with development of specific IgE by the host. These two entities, infestation and sensitization, are closely related. The

pathological findings of infested pieces of tissue suggest hyper-sensitivity mechanisms [5] and the infecting live larva is probably necessary for most of the anaphylaxis or urticaria cases to occur.

The first cases of allergy to *A.s.* in Spain were described in 1995 [6], and many more began to be diagnosed shortly thereafter [7, 8]. At present, they make up no less than 10% of the anaphylaxis cases in our country and 10% of the acute urticarias in the emergency services [9]. In the Madrid population, allergy to *A.s.* has been detected in 5.7% of the patients who attend the allergy out-patient clinic for any other reason [10]. In studies carried out by the Spanish Society of Allergology and Clinical Immunology, it has been observed that 38% of the Spanish population who have developed an urticaria present specific IgE to *A.s.*

Diagnosis of this type of allergy is based on the clinical history and demonstration of specific IgE. However, the allergic symptoms, as opposed to other cases, can appear several

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hours or even days after the intake of fish and thus, it is difficult to associate the symptoms with the food in many cases. The skin tests and the determination of specific IgE to *A.s.* with whole-body antigen have been standardized [11]. With these tests, an important prevalence of subclinical sensitization has been detected in Spain. In Madrid, where eating raw anchovies marinated in vinegar is common, a 23% prevalence of subclinical sensitization has been estimated in the patients who attended an allergy service [10].

Subclinical sensitization occurs mainly in people who have had contact with the larvae, without having developed symptoms. Contact with this parasite in European countries is very high, probably because the fish banks from the North Atlantic Ocean are frequently contaminated [12, 13] and the fish intake, especially in Spain, is high (89 g/person/day) [14]. Certainly, a small proportion of cases reflect cross-reactivity with other parasites [15] or other allergens derived from chironomids, shellfish [16] and other arthropods [17], given that the tropomyosin is also an allergen described in *A.s.*

Many studies have been carried out over these years in an attempt to distinguish patients with clinical and subclinical sensitization. Certain IgE immunoblotting patterns have been reported to be associated with patients who had allergy, as opposed to those who were apparently asymptomatic carriers of specific IgE to *A.s.* [18]. However, there is a high overlap in these patterns, and this technique did not provide definitive answers to help diagnosis. Monoclonal antibodies have been raised against specific allergens that are mainly recognized by allergic patients [19] and the allergens Ani s 1 [20, 21] and Ani s 2 (tropomyosin) have been cloned. However, none of these techniques are readily available for use in the healthcare practice. *In vivo* 'gold standards' have been studied to differentiate these patients through oral challenge with lyophilized larvae [22] and parasitic antigens [23]. The results of these challenges, performed in subjects who are clearly allergic, have been surprisingly negative. These findings can suggest that the clinically important allergens are those released by the live larva during the gastrointestinal infestation and thus, consumption of the dead larva does not cause allergic reactions. In this study, the allergens secreted by the parasite have been partially characterized, their potency has been compared with the somatic antigens and their clinical importance has been studied.

Materials and methods

Patient data and sera

A total of 10 patients (six women and four men) were selected (Table 1). Their mean age was 47 years (range: 35–62 years),

they had presented one or more episodes of urticaria ($n = 4$) or anaphylaxis ($n = 6$) several hours after having eaten fish (anchovies marinated in vinegar, raw sardines, grilled shrimp or fried hake) and had been diagnosed of allergy to *A.s.* They had positive skin tests to *A.s.* (IPI SA, Spain) and positive determination of specific IgE (CAP Pharmacia[®], Uppsala, Sweden) to *A.s.* with a mean of 67.26 ± 26.75 kU/L. The allergologic study with the fish or shellfish involved in each case was negative. These patients did not have parasitic eggs in faeces.

A specific written authorization was obtained from all of them to perform the prick tests and challenge studies with *A.s.* proteins.

Individual sera were used in the experiments, and also a pool of the 10 sera in equal volumes.

Preparation of *Anisakis simplex* extracts

Live larvae extracted with forceps from parasited fish were provided by the Health Care and Social Services Council of Madrid through the City Food Hygiene Laboratory. The larvae identified by experts were extensively washed in tap water and left to rest in phosphate buffer for 24 h, discarding the supernatant at 24 h to avoid contamination with fish proteins.

Two types of allergenic extracts were prepared with this material:

(a) *Excretory/secretory (E/S) extract*: The larvae of *A.s.* were cultured following the Raybourne et al. procedure [24]. They were introduced into sterile flasks with phosphate-buffered saline (PBS) at 0.5 mL/larva, and glucose (1%), gentamycin (150 µg/mL) and vancomycin (10 µg/mL) were added. The culture was conserved at 37 °C in 5% CO₂ for nine consecutive days. The medium was recovered every three days, adding new buffer. It was verified that during this time most of the larvae remained alive and had good mobility. The medium recovered from the first three days was called phase 1 (P1), that of days 4–6, phase 2 (P2) and the last, phase 3 (P3). They were concentrated by pressure in a concentrator (Amicon, Millipore Corporation[®], Bedford, USA) through 3000 Da pore size membranes. They were centrifuged at 40 000 g for 20 min and the supernatant was dialysed against PBS for 24 h with continuous agitation in a cold room to eliminate any remains of the antibiotics. Finally, each extract was passed through a 0.22 µm filter (Milled[®]-GS, Millipore SA, Moslem, France) and conserved until use in a freezer at –40 °C. In many experiments, a mixture of three extracts (complete extract) was used, which we called E/S.

(b) *Somatic (S) extract*: A modified protocol of Desowitz et al. [25] was followed. The *A.s.* larvae were frozen in liquid nitrogen, and were then ground in PBS in a homogenizer, and

Table 1. Clinical characteristics of the 10 studied patients

Patients #	1	2	3	4	5	6	7	8	9	10
Symptoms	URT	ANA	URT	URT	ANA	ANA	ANA	ANA	URT	ANA
Sex	F	F	M	M	F	M	F	M	F	F
Age (years)	37	54	45	35	40	52	42	45	48	62
AsIgE (kU/L)	54	> 100	35.8	> 100	74.5	44.9	18.7	95.2	33.3	> 100

URT, urticaria; ANA, anaphylaxis; AsIgE, specific IgE to *Anisakis simplex*.

sonicated on ice at 100 W for five periods of 30 s. The extract obtained was shaken in the cold room for 1 h and then centrifuged at 40 000 *g* for 20 min. The supernatant was finally processed, following the previously described steps with the E/S extract (dialysis, filtration and freezing).

The aliquots to be used at *in vivo* tests were analysed by microbiologic techniques to verify their sterility. The protein content of the extracts was analysed using the Bradford technique [26].

Skin tests

Prick tests were performed with the three fractions of the E/S extract, the complete E/S extract and the S at several concentrations. Tests were considered positive when the largest diameter of the wheal was equal to or greater than 3 mm, as long as the negative control did not present a reaction. The surface of the wheals and erythema corresponding to protein concentrations that were equal for all the extracts was compared. Controls were performed with E/S and S extracts in 10 healthy subjects with negative skin test to the commercial extract of *A.s.* and serum IgE against this antigen.

ELISA

Different protein concentrations of antigen were applied, in duplicate, in 96-well polystyrene plates (Costar®, Corning Inc, NY, USA), for 18 h at 4 °C. The wells were blocked with skimmed milk at 5% in casein–thimerosal buffer (CTB) for 1 h at 37 °C. After washings with CTB, the patient's serum, to be tested in each experiment, was added in PBS, and then human anti-IgE conjugated with peroxidase (Sigma Chemical®, St Louis, USA) in CTB for 1 h at 37 °C. Extensive washings were performed with CTB between each of the steps. Finally, o-phenyldiamine (1 mg/mL) in phosphate–citrate buffer and H₂O₂ at 15% was added. The reaction was stopped with SO₄H₂ · 2N. Optical density was measured at 495 nm.

SDS-PAGE and blotting

Apparent molecular weights (MWs) of the proteins of the extracts were determined by a 15% SDS-PAGE according to standard procedures [27]. In each gel lane, 4.4 µg of protein of extract was added (unless otherwise indicated) and then separated protein bands were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon®-P, Millipore Corporation, Bedford, USA), in a semi-dry blotting system for 50 min. Finally, the proteins were stained with Amido Black.

Immunoblotting

The membranes, with transferred parasitic proteins, were blocked with milk at 5% in Tris–HCl buffered saline solution (TBS) for 1 h. Afterward, they were incubated with continuous shaking at room temperature with the patient's serum (1/5) in TBS–Tween 20 (TTBS) for 18 h. The membranes were then incubated with anti-human-IgE conjugated with peroxidase in TTBS for 1 h at room temperature. Washings with TTBS with milk at 1% were performed between each step. Finally, the membranes were incubated in chemiluminescent

substrate (Supersignal® West Pico Chemiluminescent Substrate, Pierce®, Rockford, USA) for 5 min, and then they were exposed to radiographic films.

Immunoblotting inhibition

Specific inhibition studies (immunoblotting) were carried out by pre-adsorption of patients' sera for 1 h with equal amounts of inhibitor extracts (E/S or S). These studies were performed to identify the common antigens of the E/S extract with the S.

Single-blind placebo-controlled oral challenges

Oral challenge tests were performed in each patient with increasing doses of E/S antigen. The study begun with the administration of the placebo (lactose) and then with the buffer solution used as a culture medium of the larvae. Once the vehicle was tolerated, increasing doses of the E/S extract were administered orally, at intervals of 2 h, reaching an accumulated dose of 479.44 µg, which would be equivalent to the protein production of approximately 100 larvae of *A.s.* The study performed was single blind. The protocol was revised and approved by the ethical committee of Gregorio Marañón University Hospital of the 'Comunidad Autónoma de Madrid': CAM 08.6/0010/2000.

Studies on resistance of parasitic antigens to acid pH and pepsin

The E/S extract was incubated with pepsin from the porcine gastric mucosa (Sigma Chemical®, St Louis, USA) at different concentrations of 0.1, 0.01 and 0.001 mg/mL for 2 h and was then dialysed against the carbonate/bicarbonate buffer. The antigen was used to perform the ELISA for specific IgE detection with a pool of 10 sera.

HCL 13 M was added to two aliquots of the E/S extract until pH: 2 and 4, respectively, were obtained and it was maintained for 2 h. Afterward, the extract was dialysed against carbonate/bicarbonate buffer to coat the plate. The 10 individual sera were checked for specific IgE by ELISA.

The protein concentration of E/S extract used to coat the plate was 10 µg/mL in both assays.

The ANOVA (repeated measures) test was used for statistical analyses.

Results

Protein and allergenic pattern

The final concentration of the P1, P2, P3, E/S and S extracts was 0.33, 0.074, 0.063, 0.11 and 0.6 mg/mL respectively. In the nine days of culture, a mean of 4.4 µg of protein was produced for each larva of *A.s.* Sixty-eight percent of the total protein production was obtained during the first three days and 17% and 15% in phases 2 and 3, respectively. Figure 1a shows the principal proteins produced during the different culture stages. Certain proteins were only excreted during the first days of the culture and decreased over time; however, most of them were secreted during the whole culture.

IgE binding components to S, E/S extracts and their fractions were determined by immunoblotting and were

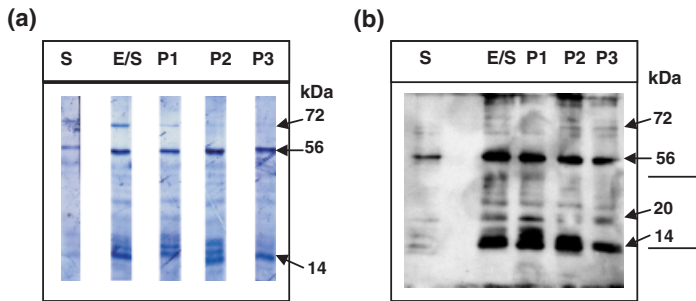


Fig 1. Protein and allergenic pattern of the *Anisakis simplex* extracts. SDS-PAGE protein and IgE binding patterns of S, E/S, P1, P2 and P3 extracts stained with amidoblack (a), or probed with serum pool (specific IgE) (b), respectively. Molecular weight is indicated on the right of each figure.

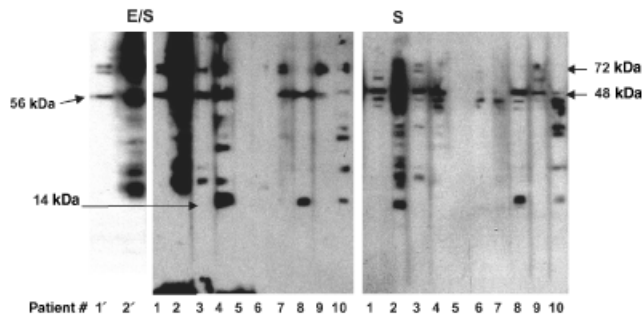


Fig. 2. Antigenic pattern. IgE immunoblot with excretory/secretory (E/S) and somatic (S) extracts. Each lane [1–10] has been probed with the serum of each patient. Immunoblot obtained with patients # 1 and 2 with less exposure time (1' and 2').

compared using a pool of the sera from the 10 patients (Fig. 1b). Most of the antigens to which the patients were sensitized were secreted during the entire culture. However, an exclusive production of allergens of 17–20 kDa could only be observed during the first three days of the culture.

Individually, the antigenic pattern of the extracts E/S and S was determined in each patient (Fig. 2). In this case, 4.4 µg and 12 µg of protein per lane of the E/S and S extracts, respectively, were applied. The allergenic pattern found in each patient was different with E/S or S extracts. Although allergens having equal MW were found in the two extracts, the patients' sera did not react equally to both of them. Patient 4 reacted to a 14 kDa present in E/S extract and did not detect the 14 kDa protein revealed by other patients' sera in the S extract. Multiple different allergens were present in the two extracts.

The antigens recognized mostly by these patients (> 50%) were 72 and 56 kDa in the E/S extract and 56, 48 and 43 kDa in the S one. We did not find a common allergen to all the patients in either extract.

Antigenic potency

Serum-specific IgE to the S, E/S extract and the three fractions of the culture (P1, P2, P3) in each patient were compared by ELISA. Figure 3a shows the comparison of the absorbance at 495 nm between the S/E and S extracts. This assay was performed with each of the 10 sera and four different extract concentrations and the mean was plotted. The E/S extract was shown to be 5.8 ± 0.48 (standard error, SE) times more potent than the S extract.

The *in vivo* parallel study (skin tests) was similar. The 10 patients had a positive reaction in the S, E/S extracts and fractions. These extracts were tested at different concentrations

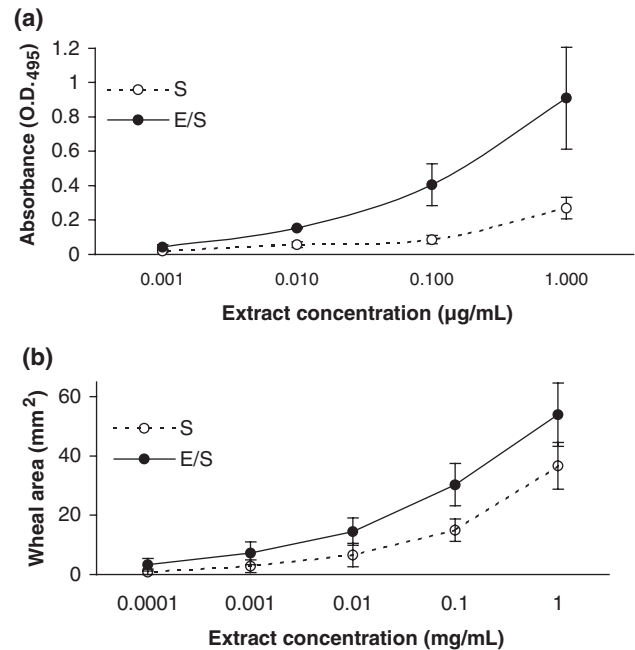


Fig. 3. Antigenic potency. ELISA optical density absorbances at 495 nm of specific IgE with somatic (S) extract and excretory/secretory (E/S) extract coated with protein concentrations: 1–0.001 µg/mL (mean value of the results obtained with 10 individual patients) (a). Prick test: area in mm² of the wheal obtained with different E/S and S extracts (1–0.0001 mg/mL, mean of 10 patients) (b).

and the area of the wheal obtained was measured (Fig. 3b). The wheal obtained with the E/S extract was 5.89 ± 0.23 (SE) times greater than that produced by the somatic antigen. The results obtained when the area of erythema was measured in each patient revealed similar results (the E/S extract caused a bigger area of erythema than the S extract, data not shown). The skin tests were negative in the 10 control patients.

When the fractions were compared, the pool sera were tested.

It was observed that the IgE binding decreased during the culture. The ELISA readings revealed a 25.1% and 42.1% decrease in the absorbance in P2 and P3 compared with P1, respectively, in spite of comparing them with an equal protein concentration (figure not shown).

Cross-reactivity studies

A cross-reactivity study was performed between the E/S and S extracts with a pool sera formed by equal volumes of each of the 10 patients who participated in the study to observe the

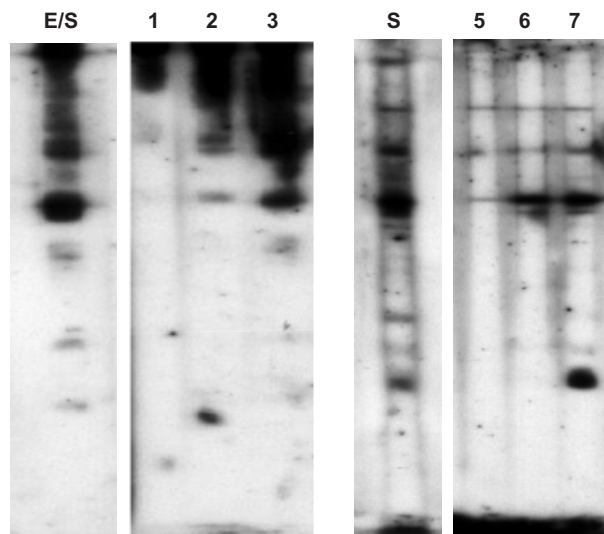


Fig. 4. Cross-reactivity between excretory/secretory (E/S) and somatic (S) allergens. IgE immunoblot inhibition. The E/S extract in the solid phase (E/S) inhibited by the S extract at different dilutions 1/10 (1), 1/100 (2) 1/1000 (3). The S extract in solid phase (S) inhibited by the E/S extract at a dilution of 1/10 (4), 1/100 (5), 1/1000 (6).

common allergens (Fig. 4). In this study, 4 µg and 12 µg of protein/lane were applied for the E/S and S extracts, respectively, to be able to detect most of the antigens. Low and medium MW bands entirely disappeared upon cross-immunoblotting inhibition, while some high MW components remained unchanged in both extracts. Although many common antigenic determinants were present in both extracts, the high MW molecules did not seem to have an allergenic relationship. No quantitative analysis of this inhibition was performed.

Oral challenge with E/S extract

The controlled oral administration of the E/S extract was negative in all the patients, reaching a total accumulated dose of 479.44 µg. Challenges with the negative control (placebo and vehicle) were also negative.

Studies of antigenic sensitivity to pepsin and acid pH

The increasing concentrations of pepsin (0.001, 0.01 and 0.1 mg/mL) produced a loss of binding of the specific IgE to the E/S antigen of 21.3% (NS), 45.4% ($P = 0.01$) and 85.8% ($P = 0.03$), respectively (Fig. 5a).

The acid pH did not affect the overall binding to IgE to the E/S extract. It decreased by 15.23% and 19.96% at pH 4 and 2 (mean of 10 patients, $P = 0.168$ and 0.06, respectively). We performed this particular study in every patient to look for individual responses. A decrease in binding was only observed in the case of the first two patients (Fig. 5b).

Discussion

The *A.s.* larvae secrete proteins into the medium called E/S antigens. In this study, we have found that these molecules

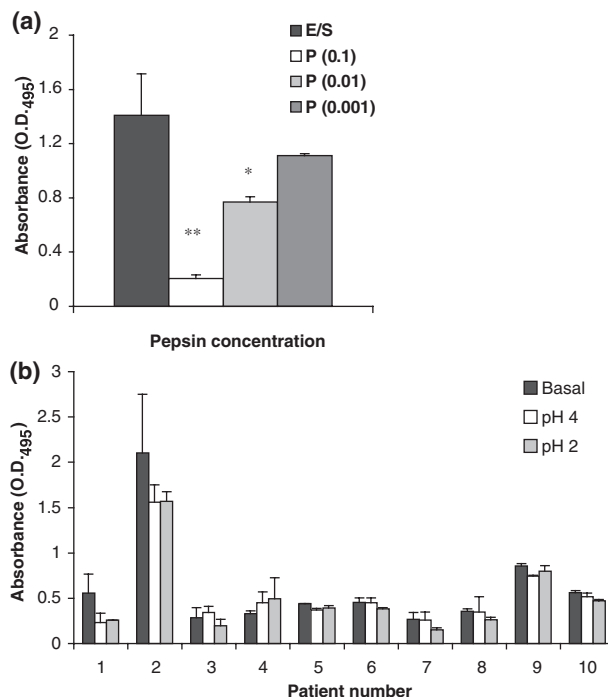


Fig. 5. Resistance of excretory/secretory (E/S) allergens to pepsin and low pH IgE ELISA. (a) Sensitivity to pepsin: basal E/S level (BASAL), E/S treated with different concentrations (0.1, 0.01, 0.001 mg/mL) of pepsin (pool of sera of 10 patients, mean $\pm$ SD). ** $P < 0.001$, * $P < 0.03$. (b) Study of antigenic sensitivity to acid pH: basal E/S level (BASAL), and treated with pH 4 and 2 results with serum of each patient [1–10].

are much more potent allergens than those present in the S extracts and presently used in the diagnosis. The E/S extract is more potent because it is able to bind more to serum IgE and elicit a bigger skin test at the same protein concentration. This could be caused because there is a higher relative concentration of *A.s.* allergens in this extract or because the non-shared antigens (maybe the high MW molecules) present in E/S extract have more affinity to specific IgE. As yet, they do not elicit a skin reaction in negative patients.

The proteins and especially the allergens released into the medium decreased noticeably as the days of the culture increased. It is possible that the conditions of this culture, with glucose as the only nutrient, are not sufficient to maintain the production capacity of the larvae at 100%. These conditions were chosen based on the Raybourne et al. [24] cultures in order to avoid the presence of fetal calf serum that could interfere in the *in vivo* or *in vitro* studies in allergic patients. Most of the allergens of the E/S extract, although in less amounts, were released during all the days of the culture and only some were exclusively secreted in the first days.

Our patients seemed to be sensitized to a great number of these proteins present in both the E/S as well as in the S extract. There was much variability of recognition of the allergens by the different patients in both cases. In these patients, we did not detect any allergen invariably recognized by all of them. Most of the patients recognized different allergens in both extracts, yet, most of the antigenic determinants present in the proteins of the S extract might be represented in the E/S protein group and vice versa, since

we obtained a high inhibition between them in the IgE Immunoblot. Given that this study was performed with a mixture of sera from all the patients, it is possible that some of the antigens recognized by a small percentage of the patients were not detected in the inhibition study. Thus, it might be interesting to mix both types of extracts for diagnostic purposes in order not to miss some specificities.

The data obtained in the challenge tests of the antigens have been surprising. The E/S extract is much more potent; however, all the challenges performed with the amount of protein secreted by 100 larvae in these patients were not capable of causing an allergic reaction orally. The sensitivity studies to gastric pepsin can explain these results. On contact with pepsin, these proteins decreased or lost their allergenicity. This, with the fact that the reactions occur some hours to some days after the ingestion of fish in many patients, could reinforce the theory that the presence of the live larva and the injection of their secretion through the mucosa are essential to cause allergic reactions in most of the patients.

None of these patients developed oral allergy syndrome either; maybe salivary enzymes have the same inhibitory activity or there is not enough time in the mouth for these allergens to cause allergic reactions.

At present, most of the allergic patients are allowed to eat fish that, either because it has been frozen in ideal conditions, or because it has been very well cooked, only contain dead larvae. Most of these patients are tolerating fish [28, 29], avoiding an important problem in their dietary habits and that of their family. There is still doubt in a small percentage of patients who, according to some authors, develop allergic reactions after eating fish carrying dead larvae, as is the case of canned fish that do not need refrigeration. These patients are probably sensitized to somatic proteins or those secreted in the fish by the larva, but resistant to the gastric conditions, and thus they should avoid marine fish in their diet.

There are other reasons that could justify these negative challenges and explain the difficulty that we are having in finding clinical reference tests for the diagnosis of these patients. Allergy to *A.s.* behaves differently from the classic food allergy, and the study parameters and diagnosis should also be different. When a person is infected by a parasite, it develops parasite-specific IgE and many other IgE molecules (a polyclonal response) that may compete for the binding to their receptors on mast cells. A big polyclonal IgE response may not let two *A.s.*-specific IgE molecules be close enough to generate a reaction. The negative controlled challenge, in some cases, may only be circumstantial. The relative production of the immunoglobulins (parasite specific vs. parasite non-specific IgE or other immunoglobulins such as IgG or IgM) could determine the development of the reaction and/or its intensity. The identification of a specific immunoglobulin pattern could be helpful to detect the so-called 'subclinical sensitization'. Most of the allergic patients report urticarial or anaphylactic reactions once or twice a year, in spite of the probably frequent contact with the parasite.

In conclusion, the *A.s.* parasite secretes proteins with great allergenic potency, which should be present in the extracts used for the clinical diagnosis. The purification of E/S and somatic proteins and their use in the diagnostic methods can complement the present diagnostic methods and help to understand this interesting cause of allergic reaction.

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