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Evaluation by the skin prick test of *Anisakis simplex* antigen purified by affinity chromatography in patients clinically diagnosed with *Anisakis* sensitization

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Abstract

Anisakis simplex crude extracts (CE) (IPI, ASAC and ALK-ABELLÓ), *A. simplex* larval antigens purified using a column of IgG anti-*A. simplex* (PAK) or a column of IgG anti-*Ascaris suum* (PAS), antigen eluted from columns of IgG anti-*A. suum* (EAS) and an *A. suum* adult CE were assayed by the skin prick test. Thirty percent of assayed patients showed a negative reaction in the *Anisakis* skin prick test. Of 70% positives, two patients had a weal greater than that produced by histamine with the *A. simplex* extract from ABELLÓ and IPI. The *A. suum* skin prick test was positive in 35% of patients, with a lower reaction than that observed with the *A. simplex* extract from IPI in 57% of the sera and a higher reaction in 28% of the sera. All patients with positive reactions with the crude extract also showed positive weals with the two purified antigens, PAK and PAS. All patients, except three, with a reaction to *A. suum* antigen, were positive to the EAS antigen. In five patients the weal size produced by PAS was greater than that observed with PAK, whereas in another six patients the contrary was observed. Only one of these six patients did not react to EAS antigen, coincident with the patient showing only a slight increase (7%) in the weal size induced by PAK vs. PAS. When the EAS antigen was tested on patients positive to both PAK and PAS, six patients presented a weal size of $>30\%$ and only three patients who were positive to PAS did not react to the EAS antigen. These three patients were also negative against the *A. suum* CE. Purification by affinity chromatography eliminates from the PAS antigen the proteins responsible for producing cross-reactions with *Ascaris* (present in the EAS antigen).

Introduction

Although the first Spanish case of sensitization attributed to *Anisakis simplex* (Ascaridida: Anisakidae) was only reported eight years ago (Audícana *et al.*, 1995), several other such cases have been observed and investigated since then (Fernández *et al.*, 1996; del Pozo *et al.*, 1997; Estrada & Gozalo, 1997; García *et al.*, 1997, 1999; Montoro *et al.*, 1997; Anibarro & Seoane, 1998; Armentia *et al.*, 1998; Cuende *et al.*, 1998; Daschner *et al.*, 1998, 2000; Fraj *et al.*, 1998; Gómez *et al.*, 1998; Rosel *et al.*, 1998; Alonso *et al.*, 1999; Mendizabal, 1999; Domínguez

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et al., 2000, 2001; Esteve *et al.*, 2000; Perteguer *et al.*, 2000). Since the Spanish consume large quantities of fish (78.2 g per person per day), the real prevalence and incidence of anisakiasis in Spain may be much greater than indicated by these reported cases.

Several methods have been used for the immunodiagnosis of anisakiasis, including the complement fixation test (Daniels, 1962), immunofluorescence test (Ruitenberg, 1970), immunoelectrophoresis (Suzuki, 1968), radioallergosorbent test (Desowitz *et al.*, 1985), enzyme-linked immunosorbent assay (ELISA) (García *et al.*, 1996), CAP-fluoro enzyme immunoassay (CAP-FEIA) (del Pozo *et al.*, 1996), and immunoblotting (Akao *et al.*, 1990). However, cross-reactions can appear between ascaridoid nematodes that diminish the potential diagnostic value of these techniques and the antigens used (Suzuki, 1968; Desowitz *et al.*, 1985; McWilliams *et al.*, 1987; Kennedy *et al.*, 1988; Christie *et al.*, 1993; Zarnowska & Jastrzebska, 1994; Cuéllar *et al.*, 1995; Iglesias *et al.*, 1996; Nunes *et al.*, 1997; Fernández *et al.*, 1998; Yahiro *et al.*, 1998; Daschner *et al.*, 2000). Lorenzo *et al.* (2000) showed the role of sugar epitopes as the cause of false-positive results in serodiagnostic assays.

The skin prick test is normally performed for diagnosing human anisakiasis in Allergy Services (del Pozo *et al.*, 1996; Scala *et al.*, 2001). To improve the specificity and sensitivity of this technique, *A. simplex* antigens prepared by affinity chromatography were assayed in the present study of the skin prick test using human patients.

Material and methods

Patients

Twenty patients (13 females and 7 males, aged between 18 and 81 years (49.0 ± 20.4) and residents of Madrid) were studied at the Immunology and Allergy Service of the Hospital del Aire (Madrid). These patients presented allergic and/or gastrointestinal symptoms (anaphylaxia, acute and chronic urticaria, antral oedema, abdominal pain, appendicitis, intestinal adhesions, intestinal obstruction) closely related to fish ingestion. The concentration of circulating anti-*Anisakis* IgE was measured by means of Pharmacia CAP System RAST FEIA (Pharmacia AB, Uppsala, Sweden). The results of this fluoroimmunoassay were reported in kU/l and converted to CAP 'scores' of 0 (<0.35), 1 (0.35–0.7), 2 (0.7–3.5), 3 (3.5–17.5), 4 (17.5–50), 5 (50–100) and 6 (>100).

Parasites

Third stage larvae (L3) of *A. simplex* were collected manually from the viscera, muscle and body cavities of naturally infected blue whiting (*Micromesistius poutassou*) and carefully washed in water. Specimens of *Ascaris suum* were obtained from natural infections of swine (Rodero *et al.*, 2001).

Antigens

For preparation of the crude extract (CE) of *A. simplex*, L3 were placed in PBS at 4°C, homogenized in a hand-operated glass tissue grinder, followed by sonication for 10 s with a Virsonic 5 (Virtis, New York, USA) set at 70% output power. The homogenate was extracted in PBS at 4°C overnight and subsequently delipidized with n-hexane and centrifuged at 8497 *g* for 30 min at 4°C (Biofuge 17RS: Heraeus Sepatech, Gmb, Osterode, Denmark). The supernatant was dialysed overnight in PBS at 4°C (Rodero *et al.*, 2001). Crude extract (CE) antigen from *A. suum* adults was obtained using a modification of the method of Welch *et al.* (1983) by homogenization and extraction in PBS at 4°C overnight (instead of an ultrasonic burst). The protein content was estimated by the method of Bradford (1976) and then frozen at −20°C until needed (Rodero *et al.*, 2001).

Hyperimmune sera

New Zealand rabbits, about 3 kg in body weight, were immunized intramuscularly with larval *A. simplex* or adult *A. suum* CE antigen as multiple 3 ml doses of antigen in Freund's Complete Adjuvant (FCA) (1000 μg ml^{-1} in final volume) given weekly for 3 weeks (Rodero *et al.*, 2001).

Purification of Anisakis simplex *CE products by affinity chromatography*

Protein A-Sepharose CL-4B bead (Pharmacia Biotech) columns were prepared according to the manufacturer's instructions. Rabbit anti-*A. simplex*, or -*A. suum* antibodies, in sample buffer (0.5 M Tris, 0.5 M NaCl, pH 8.0) were loaded into the columns. Fractions of 1 ml were then collected. Unbound immunoglobulins were then eluted with glycine buffer (0.2 M glycine, 0.5 M NaCl, pH 2.8). Fractions were collected onto 100 μl of collection buffer (Tris-base 1 M, pH 8.5) and read on a spectrophotometer at A_{280} for calculating the rabbit IgG concentration. Protein A affinity isolated IgG anti-*A. simplex*, at a concentration of 5 mg ml^{-1} in $NaHCO_3$ 0.1 M with NaCl 0.5 M (pH 8.5) were coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions (Pharmacia Biotech). *Anisakis simplex* CE antigen in sample buffer was loaded into the column and incubated for 3 h at room temperature. Fractions of 1 ml were then collected. Unbound antigens were washed with buffer and bound antigens were then eluted with glycine buffer followed by 50 mM diethylamine in saline, pH 11.5 collecting into glycine to neutralize eluted fractions. Fractions were read at A_{280}. The same procedure was carried out using columns prepared with rabbit IgG anti-*A. suum.*

Prick test

Two commercial extracts of *A. simplex* (1 mg ml^{-1} from International Pharmaceutical Immunology, ASAC Pharmaceutical International, Alicante, Spain; and 2 mg ml^{-1} from ALK-ABELLÓ, Madrid, Spain) were used to investigate the sensitization of each patient to the parasite. Likewise, affinity chromatography purified antigens were used at a concentration of 1 mg ml^{-1}: *A. simplex* CE antigen eluted from a CNBr-activated Sepharose 4B coupled to rabbits immunized with *A. simplex* CE antigen (*A. simplex* PAK antigen); *A. simplex* PAK antigen after loading into a CNBr-activated

Sepharose 4B coupled to IgG from rabbits immunized with adult *A. suum* CE antigen (*A. simplex* PAS antigen); and *A. simplex* PAK antigen eluted from the anti-*A. suum* column (*A. simplex* EAS antigen). An *A. suum* adult CE antigen was used for the study of cross-reactivity. All the antigens were tested using prick-tests on the skin of the volar forearm and 15 min later, the formation of a weal measuring at least 3 mm in diameter was considered a positive result. Histamine (10 mg ml^{-1}) and saline solution were used as positive and negative controls, respectively. Mean values were obtained for weal areas, following scanning of weals, which were vectorized and, subsequently, these areas were calculated using the SuperCAD 2000 software expressed in mm^2.

Results and Discussion

Only 30% of assayed patients were negative to commercial extracts (ALK-ABELLÓ and IPI) and none of these patients showed positive results with the other antigens assayed. Only one patient who had been positive to IPI and ALK-ABELLÓ extracts, was negative to the other antigens assayed (PAK, PAS, EAS and AS). The *A. suum* skin prick test was positive in 35% of patients, with a lesser reaction than that observed with the *A. simplex* extract from IPI in half of the sera (57%), higher in 28% and similar in 14% of them. In five patients the weal size produced by PAS was greater than that observed with PAK and in eight patients the weal size was smaller. Only three of the patients that were positive to PAK and/or PAS antigen were negative to EAS and AS antigens (table 1).

For each patient showing positive results using the *Anisakis* prick extract, the percentage of the weal size was determined after the administration of the different antigens. The size of the weal produced by the histamine was considered as 100% (table 2).

Currently, the skin prick test is usually performed to diagnose human anisakidiasis in almost every allergy services. The aim of this work was to assay the specificity and sensitivity of larval *A. simplex* CE antigen purified by means of affinity chromatography by using the skin prick test on human patients.

The first purification step consisted of the binding of larval *A. simplex* antigen to homologous antibodies from rabbits experimentally immunized with this antigen and eluted from the column in order to eliminate cross-reactivity of the antigen with bacteria or viruses (Su & Prestwood, 1990). Moreover, allergenic cross-reactivity between third stage larvae of *Hysterothylacium aduncum* (Rudolphi, 1802) and *A. simplex* has been reported by Fernández *et al.* (1998) and cross-reactivity between IgE binding proteins from *Anisakis*, *Daphnia*, chironomid spp., Atlantic shrimp (*Pandalus borealis*) and German cockroach (*Blattella germanica*) by Pascual *et al.* (1997). Cross-reactivity was also observed in *Anisakis*-infected sera when adult worms of *Gnathostoma doloresi* were used as antigens (Anantaphruti, 1989). Cross-reactive specific IgE against *Ascaris lumbricoides* and *Echinococcus granulosus* was detected in gastro-allergic anisakiasis patients (Daschner *et al.*, 2000).

First of all, the reactions with crude extracts of *A. simplex* were determined. The *A. simplex* prick tests were negative in 30% of the selected patients. All these patients showed

Table 1. Weal areas expressed as mm^2 in patients following administration of different antigens using the skin prick test.

Patients	H	SS	AKABELLO	AKIPI	PAK	PAS	EAS	AS
1	43.9	0	9.62	9.41	8.66	5.80	0	0
2	84.3	0	0	0	0	0	0	0
3	59.5	0	15.07	28.33	25.69	25.35	9.42	0
4	70.78	0	18.09	9.05	4.77	7.31	2.75	0
5	57.67	0	31.41	61.93	28.03	49.94	21.87	29.46
6	21.72	0	21.95	5.36	9.98	6.17	9.10	12.09
7	73.82	10.22	24.38	20.73	12.97	12.44	12.69	19.51
8	59.56	0	27.87	29.07	15.97	23.85	0	0
9	73.18	0	0	0	0	0	0	0
10	57.29	0	49.87	50.93	34.35	29.37	18.26	0
11	67.27	0	33.62	13.85	0	0	0	0
12	83.91	0	0	0	0	0	0	0
13	26.77	0	0	0	0	0	0	0
14	23.92	0	0	0	0	0	0	0
15	66.59	17.47	64.86	57.52	52.90	38.64	42.01	13.70
16	60.72	0	58.25	32.14	33.96	41.22	28.34	0
17	25.14	0	32.16	15.15	14.07	12.32	12.87	18.21
18	102.48	12.08	39.64	78.35	51.74	58.71	58.05	68.26
19	66.06	14.99	56.89	53.77	57.62	34.27	40.83	14.11
20	125.16	0	0	0	0	0	0	0

H, histamine; SS, saline solution; AKABELLO, *Anisakis simplex* commercial extract from ABELLÓ; AKIPI, *A. simplex* commercial extract from IPI; PAK, *A. simplex* CE antigen eluted from CNBr-activated Sepharose 4B coupled to rabbits immunized with *A. simplex* CE antigen; PAS, *A. simplex* PAK antigen after loading into a CNBr-activated Sepharose 4B coupled to IgG from rabbits immunized with adult *Ascaris suum* CE antigen; EAS, *A. simplex* PAK antigen eluted from the anti-*A. suum* column; AS, *A. suum* adult CE antigen.

Table 2. Percentages of weal size determined following administration of different antigens using the skin prick test.

Patients	AKABELLO	AKIPI	PAK	PAS	EAS	AS
1	21.92	21.44	19.73	13.22	0	0
2	0	0	0	0	0	0
3	25.34	47.67	43.18	42.61	15.84	0
4	25.57	12.79	6.75	10.34	3.89	0
5	54.46	107.38	48.60	86.59	37.92	51.08
6	101.07	24.71	45.99	28.43	41.92	55.70
7	33.03	28.08	17.57	16.86	17.20	26.44
8	46.79	48.81	26.83	40.06	0	0
9	0	0	0	0	0	0
10	87.06	88.91	59.96	51.27	31.88	0
11	49.98	20.59	0	0	0	0
12	0	0	0	0	0	0
13	0	0	0	0	0	0
14	0	0	0	0	0	0
15	97.40	86.39	79.45	58.03	63.09	20.59
16	95.93	52.92	55.93	67.89	46.68	0
17	127.89	60.25	55.96	49.02	51.18	72.43
18	38.68	76.45	50.49	57.28	56.64	66.60
19	86.12	81.40	87.22	51.88	61.81	21.36
20	0	0	0	0	0	0

The weal size produced by histamine was considered as 100%. AKABELLO, *A. simplex* commercial extract from ABELLÓ; AKIPI, *A. simplex* commercial extract from IPI; PAK, *A. simplex* CE antigen eluted from CNBr-activated Sepharose 4B coupled to rabbits immunized with *A. simplex* CE antigen; PAS, *A. simplex* PAK antigen after loading into a CNBr-activated Sepharose 4B coupled to IgG from rabbits immunized with adult *Ascaris suum* CE antigen; EAS, *A. simplex* PAK antigen eluted from the anti-*A. suum* column; AS, *A. suum* adult CE antigen.

gastrointestinal findings, and three of them were also pollen allergy patients. Two patients presented a weal size greater than that produced by histamine, when *A. simplex* ALK-ABELLÓ extract were administered and only one in the case of the IPI extract. The latter patient had CAP 6 when anti-*Anisakis* specific IgE was tested by the FEIA assay (data not shown). The other two cases were patients with chronic urticaria, and a negative skin prick test to pneumoallergens and CAP 3 by FEIA (data not shown). Likewise, cross-reactivity with *A. suum* was studied. Using *A. suum* crude extract, only 35% of assayed patients were positive. Of these patients, one was also allergic to fish, seafood and dry fruits, two presented acute abdomen after ingestion of anchovies with CAP 5 by FEIA and two showed chronic urticaria, and a negative skin prick test to pneumoallergens and CAP 3 by FEIA (data not shown). These reactions were lower than those obtained using the IPI *A. simplex* crude extract in 57% of patients and higher in 28.5% of patients. When cross-reacting proteins were studied (Rodero *et al.*, 2001), an immunoblotting of *A. simplex* CE antigen was carried out using sera from rabbits immunized with *A. simplex* or *A. suum*. In the case of homologous antisera, proteins of 205, 120, 66–45, 40, 14 and 7 kDa were reacted. With sera from rabbits immunized with adult *A. suum* antigen, strong reactions were observed with 205, 120, 66–45 and 14 kDa. The molecular mass of ABA-1 is controversial, as it has been previously estimated at 14,000 (Christie *et al.*, 1990), but mass spectrometry analysis indicated that there were five components of similar size, with the major species being 14,643.2 ± 1.4 Da with a high degree of similarity amongst ascaridid parasites (Christie *et al.*, 1993). Also Kennedy *et al.* (1988) observed evidence that an Mr 14,000 component of *A. simplex* has a homologue in *A. suum* and *A. lumbricoides*. Iglesias *et al.* (1996) confirmed by immunoblotting a high degree of cross-reactivity between the somatic antigens of *A. simplex* and the ascaridoids *A. suum* and *H. aduncum*, although several *A. simplex* components in the 11–18 kDa range were only recognized by sera from mice infected with *A. simplex*. Using radioimmunoassay techniques, Tanaka *et al.* (1983) observed that small amounts of a substance cross-reacting with *A. suum* protein were exhibited by *Anisakis* larvae. High concentrations of *A. suum* protein were observed in sera from patients with anisakiasis (75.2 ± 28.0 ng ml^{-1}) compared to ascariasis (64.5 ± 18.8 ng ml^{-1}).

On the other hand, the reactions with purified *A. simplex* antigens were tested. The second path of the purification consisted of eliminating the *A. suum* cross-reactive molecules. *Anisakis simplex* PAK antigen was purified from the column prepared with anti-*A. suum* rabbit IgG using affinity chromatography. After passing across the anti-*Ascaris* column, proteins of *A. simplex* antigen purified by the anti-*A. simplex* column were maintained but an increase in the intensity of the bands of 40 and 14 kDa was noted (Rodero *et al.*, 2001). After loading into a column of anti-*A. suum* rabbit IgG, unbound antigens were of 120, 66–45, 40, 31–21 and 14 kDa with elevated concentrations of these specific proteins than those observed in the non-purified samples except in the case of the 14 kDa protein. The eluted fractions, which contained cross-reacting antigens with *A. suum*, showed

bands in the molecular weight range at 40 and 14 kDa, observing in the latter higher concentrations than in the specific antigen (Rodero *et al.*, 2001).

Using the skin prick test, the three *A. simplex* antigens purified by affinity chromatography were assayed. *Anisakis simplex* CE antigen eluted from a CNBr-activated Sepharose 4B coupled to immunized rabbits. When these antigens were administered, all patients, except the pollen allergic patient, who had shown positive results with the larval *A. simplex* CE, had reactions to the two purified antigens (*A. simplex* PAK and PAS antigens) and 30% of the observed patients were negative to all antigens tested. When we previously studied differences in antigen composition prior to and after its purification across the anti-*A. simplex* column, both antigen preparations were similar. However, in 50% of patients, the weal size produced by PAK versus IPI's commercial extract was lower, and in 35% was similar. The sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) patterns of *A. simplex* CE antigen showed proteins of 205, 120, 66–45, 40, 31–21 and 14 kDa. After purification by the rabbit IgG anti-*A. simplex* column, the same proteins were observed (Rodero *et al.*, 2001) but in different proportions, and these proteins could be responsible for producing a degradation in weal size. Of the positive patients, 14% showed the same reactions for PAK and PAS antigens, 7% were negative to both purified antigens and 36% showed more reaction with PAS antigen vs. PAK indicating that a concentration of the proteins responsible for the IgE increase stimuli has been produced. In contrast, in six patients the weal sizes were small, probably due to the elimination of the *A. suum* cross-reactivity antigens (present in EAS antigen), because only one of those six patients showed no reaction with EAS antigen. This was the same patient who showed a minor increase in weal size (only 7%) and in the same manner four of these patients were also positive to *A. suum* CE antigen with size $\geq$ 20% from the histamine reaction. These facts confirmed that the purification of the *A. simplex* larval crude extracts by affinity chromatography using sera from immunized rabbits eliminates the antigenic epitopes which are the causes of cross-reactivity among ascaridoid parasites and the production of a false positive diagnosis of *A. simplex* sensitization.

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