

Original article

Usefulness of currently available methods for the diagnosis of *Anisakis simplex* allergy

Background: Serodiagnosis of anisakiosis and *Anisakis* allergy is difficult since many *Anisakis* antigens show cross-reactivity complications. In the present study, we assess the usefulness of the major immunologic methods currently available for the diagnosis of *Anisakis* allergy.

Methods: Four tests (skin prick test, CAP-FEIA system, Western blotting, and an antigen-capture ELISA using *O*-deglycosylated antigen bound by the monoclonal antibody UA3) were applied to *Anisakis*-free subjects and subjects with confirmed *Anisakis* allergy.

Results: The skin prick test, CAP-FEIA, and the antigen-capture ELISA identified *Anisakis* allergy sera with 100% sensitivity, while Western blotting showed 96% sensitivity. The antigen-capture ELISA also showed 100% specificity, but CAP-FEIA showed a specificity of only 50%. In Western blotting, none of the bands detected were specific for either *Anisakis*-free or *Anisakis* allergy subjects. The skin prick test was not applied to the *Anisakis*-free subjects, so its specificity could not be determined.

Conclusions: On the basis of these results, we suggest that the most appropriate procedure for diagnosis of *Anisakis* allergy suspected on clinical grounds is the antigen-capture ELISA using UA3, or, alternatively, a preliminary skin prick test with a positive result subsequently confirmed by UA3-ELISA.

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Key words: allergy; *Anisakis simplex*; antigen-capture; CAP-FEIA; ELISA; IgE; immunoblotting; immunodiagnosis; prick test; UA3.

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Accepted for publication 16 February 2000

In recent years, *Anisakis simplex* has been recognized as an important cause of disease in man, both as an infective pathogen and as a food-borne allergen source (1–3). Its life cycle passes through euphausiid crustaceans (the intermediate hosts), seawater fishes (the paratenic hosts), and marine mammals (the definitive hosts) (4). Generally, human beings can harbor only the L3 and/or L4 stages (5, 6), although there has been one report of an adult worm in the intestine of a woman (7). Most *A. simplex* infections of man are due to a single larva (6). Nevertheless, its allergens are highly potent, and even a single larva contains sufficient allergen to induce a reaginic antibody response in both man and animals (8, 9).

Unfortunately, however, serodiagnosis is hindered by the fact that *A. simplex*, like other members of the Anisakidae family, bears multiple antigens that may cross-react not only with antibodies to related nematodes (10–12) but also with antibodies to unrelated microorganisms, parasites, insects, and probably plants (13–16). Therefore, it is perhaps surprising that two of the methods that are currently most frequently used to determine serum IgE antibodies against *A. simplex* (CAP-FEIA [Pharmacia Biosystems, Sweden] and IgE

immunoblotting [8]), or to demonstrate an IgE-mediated mechanism – the *in vivo* skin prick test (8) – use whole antigen from the parasite, which probably induces false-positive results. Recently, we have proposed an immunologic test based on antigen-capture ELISA, using a deglycosylated parasite allergen captured by the monoclonal antibody UA3 (17), as an alternative method for anti-*Anisakis* IgG/IgE determinations (18). To date, however, there have been no comprehensive studies of the usefulness of the different diagnostic procedures available, and it is not clear which procedure offers maximum sensitivity and specificity for allergologists who need to determine anti-*Anisakis* IgE antibodies in the serum of patients with clinical suspicion of *A. simplex* sensitization.

In this study, to investigate this question, we used the four available methods to detect the presence of anti-*Anisakis* IgE in three groups of subjects:

- 1) 36 Argentinian subjects free of *A. simplex* infection but having one or more related or unrelated parasite infections
- 2) 78 Venezuelan individuals also free of *Anisakis* infection but from an area with a high prevalence of other parasite infections (Táchira State)

- 3) 24 Spanish subjects with a clear clinical history of allergy to this parasite.

The four methods used were the *in vivo* skin prick test, CAP-FEIA, IgE immunoblotting, and antigen-capture ELISA with UA3. The *in vivo* prick test was used only in the Spanish allergy population.

Material and methods

A. simplex allergy sera

Twenty-four sera from patients showing allergy to *A. simplex* were obtained from the Allergy and Immunology Department of the Santiago Apóstol Hospital (Vitoria-Gasteiz, Spain). All patients included in the study showed clinical symptoms of *A. simplex* allergy, including urticaria/angioedema or anaphylaxis within 6 h after ingestion of fish. Furthermore, all patients showed

- 1) positive reaction in skin prick test with *A. simplex* extract
- 2) presence of IgE to whole *Anisakis* antigens as measured by the CAP system FEIA (Pharmacia & Upjohn, Uppsala, Sweden)
- 3) absence of known allergy to fish or other likely agents.

CAP IgE values ranged from 3.6 (class 3) to >100 kU/l (class 6).

Argentinian population sera

The 36 sera from subjects from the suburbs of Resistencia (northeastern Argentina) were provided by Dr José Mario Alonso of the Instituto de Medicina Regional, Universidad Nacional del Nordeste, Resistencia, Chaco (Argentina). Most of these subjects had multiple parasite infections, in accordance with the fact that they live in poor hygienic conditions without adequate sanitation. These poor conditions were particularly extreme for 19.4% (7/36) of the subjects, who also had malnutrition problems. Coprologic examination (34 of the 36 patients) revealed intestinal parasites in 91% (31/34) of patients, but not in the remaining three patients.

Venezuelan population sera

Seventy-eight sera from normal blood donors (both sexes), aged 18–45 years, were obtained from Dr Fernando Merino (Departamento de Fisiología, Facultad de Medicina, Universidad del País Vasco, Bilbao, Spain). These subjects are from a population of low socioeconomic level in inland Venezuela (Táchira State, 800–900 km from the coast). Intestinal parasites were present in 100% of the population, with the parasites of the genera *Necator*, *Trichuris*, *Giardia*, and

Entamoeba all being relatively frequent. Most subjects also suffered from the common tropical parasite diseases.

Parasites

Third-stage larvae (L3) of *A. simplex* were extracted manually from the viscera and body cavity of blue whiting (*Micromesistius poutassou*) purchased in a local market.

Parasite antigens

The somatic antigen (SA) preparation of *A. simplex* was obtained as follows. Parasites were suspended in phosphate-buffered saline (PBS, pH 7.2) containing 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, Madrid, Spain), 5 mM *o*-phenanthroline (Sigma), 1 μ M pepstatin A (Sigma), 10 μ M E64 (Sigma), 3 μ M aprotinin (Sigma), and 1 mM EDTA (Sigma) as protease inhibitors; they were then ruptured by homogenization in a Braun S Potter homogenizer with a Teflon pestle followed by sonication on ice (100 W, 5 $\times$ 30 s) (Branson Sonic Power Company, Connecticut, USA). The sonified homogenate was ultracentrifuged (105 000 *g* for 60 min at 4°C) and the resulting soluble fraction stored at –30°C until use. The protein concentration of the antigen preparations was estimated by the method of Lowry et al. (19) with bovine serum albumin (BSA; Sigma) as the internal standard.

Mild alkali deglycosylation

Mild alkali treatment to cleave *O*-glycosidic linkages was performed as described by MacDonald et al. (20): 4 mg of *A. simplex* SA was dissolved in 500 μ l of 0.01 M NaOH and incubated for 24 h at 50°C.

Prick tests

Skin prick tests were as described by Del Pozo et al. (21). The tests were performed on the volar aspect of each subject's forearm with allergy pricker lancets (DHS, Madrid, Spain) loaded with 0.05 mg/ml of *A. simplex* extract. Histamine (10 mg/ml) and saline solutions were used as positive and negative controls, respectively. The response was recorded after 15 min. Patients with a wheal diameter 3 mm larger than the negative control were regarded as positive.

Total IgE determination

Total serum IgE was determined by the CAP test in accordance with the manufacturer's instructions (Pharmacia-Upjohn, Uppsala, Sweden).

Anti-*Anisakis* IgE determination: CAP-FEIA

CAP-FEIA IgE determinations were performed in

accordance with the manufacturer's instructions (UniCAP, Pharmacia-Upjohn), using the *A. simplex* antigen supplied. Results higher than 0.35 kU/l were considered positive.

Anti-*Anisakis* IgE determination: antigen-capture ELISA

Levels of IgE antibodies to UA3-recognized *O*-deglycosylated antigens of *A. simplex* were determined by antigen-capture ELISA, according to Lorenzo et al. (22). First, 100 µl of PBS containing 1.4 µg of UA3 was added to each well of an ELISA plate and incubated overnight at 4°C. After washing, either 100 µl of undiluted *O*-deglycosylated *A. simplex* SA (500 µg total protein) or 100 µl of TBS-T (Tris-buffered saline containing 0.2% Tween 20) containing 3% skimmed dry milk (TBS-T₁) was added to each well and incubated for 1 h at 37°C. After a washing step and the addition of 100 µl of pure human serum, the plates were incubated for 90 min at 37°C. The plates were then washed, and 100 µl of a mouse anti-human-IgE mAb (Ingenasa, Madrid, Spain; 1/2500 in TBS-T₁) labeled with fluorescein isothiocyanate (FITC) was added. After incubation for 1 h at 37°C and subsequent washing, bound Ig-FITC was detected by incubation first with peroxidase-conjugated rabbit anti-FITC Ig (Dakopatts A/S, Denmark; dilution 1/1000 in TBS-T; 1-h incubation at 37°C) and then with *o*-phenylenediamine (OPD; Sigma). Optical densities (OD) at 492 nm were calculated by subtracting the OD values given by the same serum in the absence of antigen. Determinations were done in duplicate. For data analysis, sera were considered positive if the OD was more than $\bar{x} \pm 4$ SD, where $\bar{x}$ and SD are the corresponding mean OD and standard deviation obtained with 25 control sera (from normal blood donors, aged 18–24 years). The standard curve was constructed by incubating ELISA plates overnight with 5 µg/well of goat anti-human IgE serum (Sigma). After washing, nonspecific binding sites were blocked by incubation for 1 h at 37°C with TBS-T₁. The plate was washed and incubated with serial dilutions of a reference serum with known IgE concentration, in each case for 1 h. The wells were then washed, and captured IgE was revealed by the procedure described above. A four-parameter logistic equation was then fitted to the plot of measured OD against known IgE concentration (kU/l), with the aid of the Fig. P (Fig. P Software Corporation, Durham, NC, USA) data-analysis package.

SDS-PAGE and immunoblotting

A. simplex SA components were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on 5–20% linear gradient gels (70 × 80 × 0.75 mm) in a MiniProtein II cell (Bio-Rad, Richmond, CA, USA), as described previously (23). After electrophoresis,

proteins were transferred at 15 V for 55 min to Immobilon-P transfer membranes (0.45-µm pore size) in a Trans-Blot SD transfer cell (Bio-Rad). The membranes were incubated with individual or pooled human sera (1/5 dilution in TBS-T containing 1% skimmed dry milk [TBS-T₂]) for 2 h at room temperature, and then with the mouse anti-human IgE mAb (1/500 in TBS-T₂) for 30 min. After washing, the membranes were incubated first with peroxidase-conjugated rabbit anti-FITC Ig (1/500 in TBS-T₂), and finally with 3,3'-diaminobenzidine tetrahydrochloride (Sigma) for visualization.

Results

Specificities of the CAP-FEIA and antigen-capture ELISA methods

The specificities of the two methods for determination of anti-*Anisakis* IgE were analyzed by comparing the results obtained for the *Anisakis*-free populations (the Argentinian and Venezuelan populations) with those obtained for the allergy population (the Spanish population). To facilitate the analysis, a calibration curve was constructed with serial dilutions of a human IgE standard (see Material and methods) (Fig. 1). Although this assay can detect as little as 0.27 kU/l of IgE (corresponding to an OD of 0.068), we used a cutoff value of 0.58 kU/l (corresponding to an OD of 0.105) on the basis of IgE determinations in sera from the Argentinian population: specifically, the cutoff value was obtained by calculating mean OD plus 4 SD for the Argentinian sera, and reading off the corresponding IgE concentration from the calibration curve.

The results obtained with the two methods are summarized in Fig. 2 and Table 2. As can be seen, both methods gave positive results for all allergy patients. As expected, absolute IgE values for most of the allergy sera were higher with the CAP method, which measures responses against whole allergens contained in the

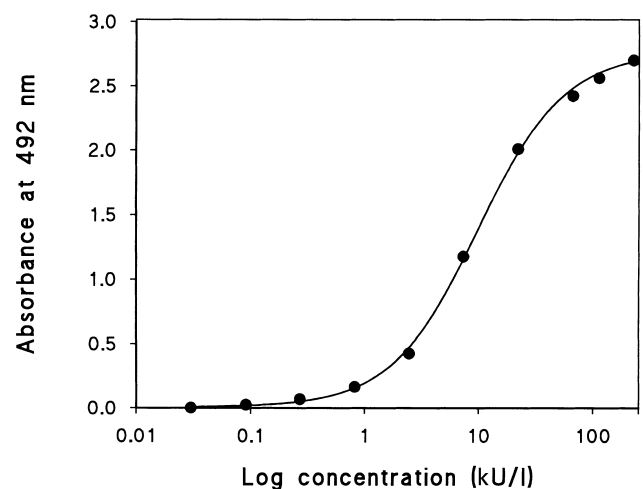


Figure 1. Calibration curve for antigen-capture ELISA.

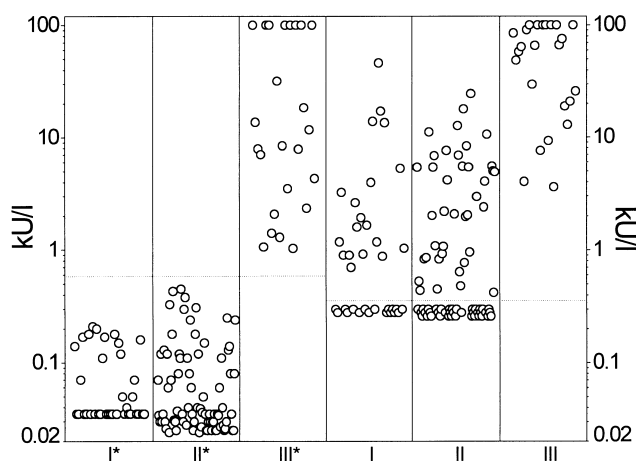


Figure 2. Anti-*Anisakis* serum IgE levels (kU/l) as measured by antigen-capture ELISA with UA3 (*) or by CAP-FEIA (Pharmacia) in 36 Argentinian *Anisakis*-free subjects (I), 78 Venezuelan *Anisakis*-free subjects (II), and 24 Spanish *Anisakis* allergy patients (III). Each point represents individual serum. Horizontal bars represent cutoff values (0.58 kU/l for ELISA, 0.35 kU/l for CAP).

parasite. This was most evident in the case of the sera showing relatively low anti-*Anisakis* IgE values: only two patients showed CAP IgE values below 5 kU/l, as opposed to eight patients when IgE was assayed by the ELISA method (Fig. 3).

By contrast, the results obtained for the *Anisakis*-free individuals clearly differed depending on the immunoassay used. Specifically, while the percentage of false-positive results obtained with CAP was 45% in the Argentinian *Anisakis*-free population and 50% in the Venezuelan *Anisakis*-free population, none of the 114 sera tested were positive by the ELISA method (Fig. 2).

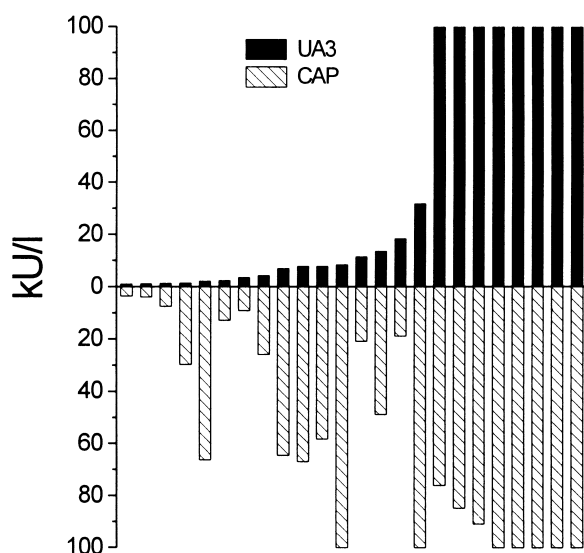


Figure 3. Anti-*Anisakis* serum IgE levels (kU/l) as measured by antigen-capture ELISA with *O*-deglycosylated UA3 (black bars) or by CAP-FEIA (striped bars) in Spanish *Anisakis* allergy patients.

Most of the false-positive CAP values were class 1 (0.35–0.7 kU/l), but 19 of the 119 subjects tested (16%) showed CAP values between 5 (class 3) and 47 kU/l (class 4).

When we compare the methods, the IgE values obtained for allergy patients match relatively well (Fig. 3): most of the patients with high IgE levels as detected by CAP also showed high IgE levels as detected by ELISA. Nevertheless, some sera (6/24) (Fig. 3) that showed less than 20% of maximal response in ELISA showed more than 50% of maximal response in CAP.

Sensitivity and specificity of Western blotting

Since Western blotting (WB) has been proposed as a more specific method than CAP for anti-*Anisakis* IgE determinations in human sera (8, 24), we investigated its sensitivity and specificity, using some of the sera from allergic and *Anisakis*-free individuals. Due to the limited volume of sera available from the Argentinian and Venezuelan subjects, the WB study was restricted to a small number of individual and pooled sera, as indicated in Fig. 4. The banding patterns were compared to those obtained with four of the *Anisakis* allergy patients and with one Spanish serum showing high levels of anti-*Anisakis* IgE (CAP=51.9 kU/l, UA3 ELISA=7.8 kU/l), but no symptoms of allergy. CAP-determined anti-*Anisakis* IgE levels in each individual or pooled serum are indicated in the legend of Fig. 4.

The results presented in Fig. 4 show the existence of a large number of bands in both the allergy and

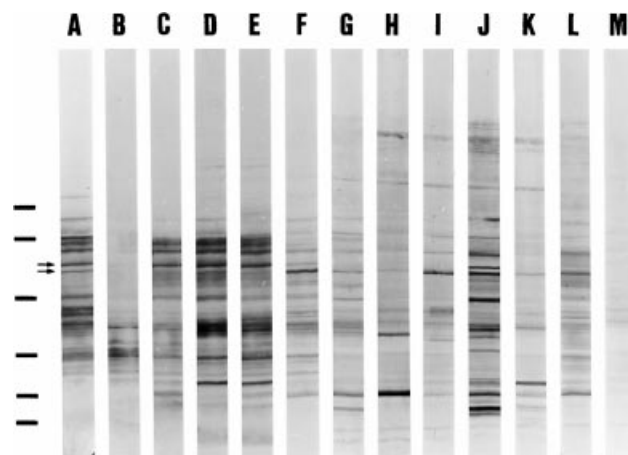


Figure 4. Western blots of *A. simplex* somatic antigens (SA) with sera from different subjects. Proteins were first separated on 5–20% polyacrylamide gel under reducing conditions. Only bound IgE was visualized. Lanes A–E) sera from allergy patients (CAP: 51.9, 26.0, 58.3, 84.8, and >100 kU/l, respectively); lane F) pooled sera of eight Argentinian *Anisakis*-free subjects (mean CAP: 12 kU/l); lanes G–L) sera of Venezuelan *Anisakis*-free subjects (in all cases, sera that tested positive in CAP, with IgE levels of 6.85, 11.2, 6.94, 24.6, 7.64, and 11 kU/l, respectively); lane M) negative control. Relative molecular masses of standard markers (97.4, 66.2, 45.0, 31.0, 21.5, and 14.4 kDa, from top to bottom) are shown on left.

Anisakis-free populations. The allergy patients showed broadly similar banding patterns (lanes A–E). In general, and as expected, band intensities correlated with anti-*Anisakis* IgE levels. However, in spite of the fact that WB was carried out after PAGE separation to improve resolution of bands, we did not detect any specific band that was recognized by all allergy sera but not by sera from *Anisakis*-free subjects. Nevertheless, two bands with molecular weights (MWs) of 51.9 and 48.4 kDa (arrows, Fig. 4) are of interest: although they were recognized by both allergy sera and sera from *Anisakis*-free subjects, the higher-MW band was preferentially recognized by allergy sera, while the lower-MW band was preferentially recognized by sera from *Anisakis*-free subjects.

Like the CAP and ELISA methods, the WB analysis showed good sensitivity, with only one false negative among the 24 allergy sera tested (Table 1).

Skin prick testing in the *Anisakis* allergy population

For ethical and practical reasons, skin prick reactions were tested only in the Spanish allergy patients. As with the CAP and ELISA methods, positive results were observed in all patients examined. The wheal diameters

Table 1. Clinical characteristics of *Anisakis* allergy patients

Patient no.	Age (years)	Symptoms	<i>A. simplex</i> skin prick wheal diameter (mm)	Total IgE	Blot patterns
1	49	U/GI	5	751	1
2	50	R/S	15	432	1
3	37	U/GI	5	271	1
4	59	I/GI	7	300	1
5	60	AE/AR	3	102	3
6	70	AE/GI/R/S	>3	448	3
7	54	U/GI	9	667	1
8	61	AE/R	>3	155	1
9	39	U/AE/R	9	354	1
10	42	U/GI	>3	1757	1
11	74	GI*	3	186	3
12	27	U	>3	304	3
13	58	U/GI	9	4179	1
14	52	U/GI/R	7	81	3
15	52	U/GI	3	3460	1
16	46	U/GI/R/S	4	217	4
17	40	U	6	432	1
18	41	U/R	8	704	1
19	63	U/AE/GI	14	397	1
20	51	U/AE/GI	13	121	3
21	50	U/GI	7	56	3
22	35	U/GI/R/S	6	92	3
23	51	U/AE	10	433	1
24	61	U/AE	>3	65	1

AE: angioedema; AI: arthralgia; GI: gastrointestinal manifestations; I: itching; R: respiratory symptoms; S: anaphylactic shock; U: urticaria.

§ Blot pattern as observed by García et al. (24).

* Diagnosed anisakiosis (two L3 found during surgery).

Table 2. Numbers of subjects from three populations testing positive for *Anisakis* allergy, as determined by CAP-FEIA, antigen-capture ELISA with UA3, immunoblotting, and skin prick tests. Percentages are shown in brackets

Population	CAP	UA3	WB	Prick
Argentinian	19/36	0/36	ND*	ND
<i>Anisakis</i> -free	(45)	(0)		
Venezuelan	39/78	0/78	ND*	ND
<i>Anisakis</i> -free	(50)	(0)		
Allergy	24/24	24/24	23/24	24/24
	(100)	(100)	(96)	(100)

ND: not determined. * However, see Fig. 4.

obtained ranged from 3 to 15 mm (Tables 1 and 2). No correlations were observed among prick response, total IgE levels, and intensity of clinical symptoms.

Discussion

Unlike gastric anisakiosis, which can be diagnosed in most cases by direct observation of the parasite by fibrogastroscopy, diagnosis of intestinal anisakiosis and/or *Anisakis*-induced allergy is often very difficult. This is mainly because healthy individuals can have high anti-*Anisakis* IgE levels in serum without clinical symptoms of allergy, while other individuals showing relatively low levels of specific IgE antibodies may have relevant clinical manifestations. Laboratory diagnosis of *Anisakis* allergy requires the development of sensitive and specific serologic tests that can overcome these problems.

Of the four methods considered in the present study, the CAP method identified allergy sera with 100% sensitivity, and IgE levels in allergy patients measured by this method were generally higher than levels measured by the other methods; however, CAP also gave an unacceptable proportion (about 50%) of false-positive results in analysis of sera from *Anisakis*-free patients. In 16% of the CAP false-positive sera, the cross-reactive IgE levels were very high (5–47 kU/l). The poor specificity of this method has not been reported previously, probably because of the inherent difficulty of obtaining sera from *Anisakis*-free populations, and this may explain why about 20% of healthy blood donors test positive by this technique (as in the prick test) (24). The epitopes responsible for the cross-reactivity of the allergens contained in crude *Anisakis* extracts have not yet been identified, but it seems likely that many molecules participate in this phenomenon. In previous studies, we have demonstrated that *Anisakis* contains N- and O-glycan epitopes borne by its glycoproteins that are able to induce cross-reactive IgE antibodies (18). The presence of IgE-inducing sugar epitopes has also been described for other common allergens (25, 26). Moreover, one or

more cross-reactive sugars present in the native antigen recognized by the mAb UA3 may also provoke false-positive results when determining IgG1 antibodies and, to a lesser extent, when testing anti-*Anisakis* IgE antibodies. Fortunately, however, these cross-reactivities can be abolished by β -elimination of *O*-glycans from the UA3-recognized antigen by very mild alkali treatment (18). Other sugar-containing allergens present in *Anisakis* extracts are alkali-resistant and therefore cannot be used for specific IgE determinations.

Another interesting aspect of the present study was the marked immunodominance of the UA3-recognized antigen (UA3R), which was recognized by 100% of allergy sera even after the β -elimination procedure. This immunodominance has also been demonstrated in mice: three of a series of five anti-*Anisakis* hybridomas developed after immunization of BALB/c mice recognized the same major bands as the mAb UA3 (17). It therefore seems likely that many of the anti-*Anisakis* IgE antibodies measured by CAP may be specific for UA3R. This would explain why positive anti-UA3R responses were obtained in the present study in confirmed-allergy sera that contained as little as 2.5 kU/l total anti-*Anisakis* IgE as measured by CAP. The high sensitivity obtained with UA3R has no precedent among the different antigens used to date in immunoassays designed to detect anti-*Anisakis* antibodies (27).

Our WB results revealed multiple allergens in *Anisakis* homogenates, but specific bands could not be resolved in a single one-dimensional run. In comparison of the two *Anisakis*-free populations, matching of many bands was observed. This is not surprising if we consider that intestinal ascaridoid parasites induce cross-reactive IgE antibodies (28), and that these and other parasitoses are very frequent in both populations. As for CAP, the presence of *N*- and *O*-glycans on these allergens could explain, at least partially, the presence of bands shared by allergy sera and sera from *Anisakis*-free subjects.

It might be expected that, as for UA3R, other allergens from *Anisakis* could be made more specific by mild alkali hydrolysis to remove *O*-glycans; unfortunately, however, this seems not to be the case, since this treatment also destroys the core protein of most of the allergens tested, invalidating them for subsequent WB analysis (18). In addition, metaperiodate treatment may also be inadequate, since periodate-sensitive and periodate-resistant allergens have recently been reported in *Anisakis* (29). In view of these results, we consider that

the view that WB is the most useful approach to diagnosis of *Anisakis*-induced allergy (24) should be reviewed.

Finally, although skin prick testing identified our allergy patients with 100% sensitivity, we did not test the specificity of this technique in our *Anisakis*-free populations, nor has specificity in *Anisakis*-free populations been investigated previously. It is generally accepted that this technique tends to provoke false-positive reactions, especially with food allergens (30); in addition, it may be supposed that, as for CAP and WB, the skin prick test will give false-positive results because of the poor specificity of the allergens used. However, since this procedure is carried out directly by the allergologist, it is a very useful tool for selecting patients with clinical suspicion of *Anisakis* allergy that can then be confirmed by more specific laboratory methods.

In conclusion, on the basis of these results, we suggest that the most appropriate procedure for diagnosis of suspected *Anisakis* allergy is the antigen-capture ELISA using mAb UA3. Alternatively, a preliminary skin prick test (high sensitivity but probably low specificity) can be performed by the allergologist, and if a positive result is obtained, the diagnosis should be confirmed in the laboratory by UA3-ELISA. The future obtainment of recombinant UA3-recognized antigen will undoubtedly facilitate the use of this analysis as a routine diagnostic tool.

Acknowledgments

We thank Dr Santiago Lojo of the Laboratorio Hospital Gil Casares (Complejo Hospitalario Universitario de Santiago de Compostela, Spain) for his help and suggestions on the standardization of the ELISA assays; Prof. Dr José Mario Alonso of the Instituto de Medicina Regional, Universidad Nacional del Nordeste, Resistencia, Chaco (Argentina), and Dr Fernando Merino of the Departamento de Fisiología, Facultad de Medicina, Universidad del País Vasco, Bilbao (Spain), for the Argentinian sera (Dr Alonso) and Venezuelan sera (Dr Merino) used in this study. This work was supported in part by grants 1028/97 from the Fondo de Investigación Sanitaria (Ministerio de Sanidad y Consumo, Spain) and XUGA20305B98 from the Secretaría Xeral de Investigación e Desenvolvemento (Xunta de Galicia, Spain). Sonia Lorenzo is supported by a fellowship from the Xunta de Galicia, Spain. Milagros García is supported by a fellowship from the Departamento de Educación, Universidades e Investigación del Gobierno Vasco, Spain.

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