

Human immunoglobulin isotype profiles produced in response to antigens recognized by monoclonal antibodies specific to *Anisakis simplex*

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Summary

Background *Anisakis simplex* is a medically important pathogen which not only causes anisakiasis but may provoke allergy reactions, ranging from mild urticaria to anaphylactic shock.

Objective To investigate anti-*Anisakis* isotype profiles in anisakiasis and *Anisakis* allergy patients.

Methods Capture ELISA techniques were used to investigate the isotype profiles of antibodies specific for two defined *Anisakis simplex* antigens, in serum from Japanese patients with confirmed anisakiasis and from Spanish patients with allergy to *Anisakis*. The antigens were 'UA2R antigens' (two proteins with M_w of 48 and 67 kDa, recognized by our monoclonal antibody UA2) and 'UA3R antigens' (two proteins with M_w of 139 and 154 kDa, recognized by our monoclonal antibody UA3).

Results Considering IgG, the two most frequent isotypes in the response to the UA2R antigens were IgG1 and IgG2, with IgG4 detected in only one case; in response to the UA3R antigens, by contrast, the two most frequent isotypes were IgG1 and IgG4 (though IgG2 remained reasonably frequent). As regards potential utility for serodiagnosis, 95% of the Japanese anisakiasis patients and 84% of the allergy patients showed detectable IgG1 antibodies to the UA3R antigens. Furthermore, all allergy patients showed IgE antibodies to these antigens.

Conclusion *Anisakis simplex* contains antigens that induce responses which are differentially regulated. Because of their immunogenicity, immunodominance and allergenic nature, we consider that the 139/154-kDa antigens recognized by our MoAb UA3 are good candidates for use in tests for the diagnosis of anisakiasis and of the allergy caused by this parasite.

Keywords: allergen, *Anisakis*, diagnosis, IgE, IgG, immunoglobulin, isotype, monoclonal antibodies, nematode

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Introduction

It has long been established that ingestion by humans of live stage-3 *Anisakis simplex* larvae present in raw or

undercooked fish may lead to infection [1]. Depending on the site of implantation of the larvae, clinical symptoms of gastric, intestinal or, less frequently, extragastrointestinal anisakiasis may arise [2,3].

More recently, however, it has also been observed that repeated exposure to *Anisakis simplex* antigens may lead to allergenic manifestations ranging from mild urticaria to

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anaphylactic shock [4,5]. Interestingly, it has also been observed that patients previously sensitized to *Anisakis* antigens, but not to fish proteins, may develop allergic symptoms after ingestion of well-cooked or even canned fish [6]. *Anisakis simplex* may thus be considered a significant agent of food-related allergy [7,8].

Anisakis simplex antigens, like those of other nematode parasites, induce high antibody levels, as is typical of TH2 T-cell subset activation. However, infection by *Anisakis* larvae is unusual for at least two reasons: (i) most cases (about 85%) are caused by a single larva [9], and (ii) only third- (L3) and fourth-stage larvae (L4) generally come into contact with the immune system, since the larvae typically die soon after infection, and very rarely reach the adult stage [10,11]. As a consequence, the host immune system typically comes into early contact with the majority of the parasite's antigens, including cuticular, excretory/secretory and somatic antigens.

To date, there have been several studies on antibody production in response to *Anisakis simplex* antigens, both in humans and experimental models, but these studies have almost exclusively used undefined antigens [12–14]. However, the development and fine-tuning of immunoassays for the diagnosis of anisakiasis and *Anisakis*-induced allergy requires analysis of the responses to individual antigens. In the present work, we analysed anisakiasis and allergy sera to investigate the isotype profiles of antibodies specific for defined parasite antigens recognized by two monoclonal antibodies (MoAbs), denominated UA2 and UA3. UA2 recognizes an epitope borne by two proteins (M_w 48 and 67 kDa, henceforth denominated the UA2-recognized antigens or 'UA2R antigens') from the somatic fraction. Additionally, UA2 recognizes two proteins (M_w 30 and 160 kDa) from the excretory–secretory fraction. UA3 recognizes an epitope borne by another two proteins (M_w 139 and 154 kDa, henceforth denominated the UA3-recognized antigens or 'UA3R antigens') from the somatic antigen fraction [15]. In addition, we have recently observed that UA3 recognizes other minor bands of lower M_w (unpublished results).

Human sera were obtained (a) from Japanese patients suffering from gastric anisakiasis, as confirmed by gastroscopy, and (b) from Spanish patients with confirmed allergy to *Anisakis simplex*, without ruling out current or previous anisakiasis.

Materials and methods

Anisakiasis patients

Thirty-seven sera from *Anisakis*-infected patients were provided by Dr N. Akao of the Department of Parasitology, School of Medicine, Kanazawa University, Japan. Of these sera, 11 had been obtained in the first 10 h following onset of

gastrointestinal symptoms; another 10 had been obtained between 24 h and 3 days after the onset of clinical symptoms. No details were available for the remaining 16 sera. All sera showed anti-*Anisakis* IgE antibodies (as measured with the CAP system FEIA, Pharmacia & Upjohn, Uppsala, Sweden) at levels ranging from 0.99 kU/L (class 2) to 88.2 kU/L (class 5), except serum #21 (0.35 kU/L, class 1) and serum #31 (<0.35 kU/L, class 0).

Allergy patients

Thirty-seven allergic sera were obtained from the Allergy Department of the Hospital Santiago Apóstol (Vitoria-Gasteiz, Spain). All patients included in the study showed clinical symptoms of *Anisakis simplex* allergy [7], including (a) urticaria/angioedema or anaphylaxis within 6 h after ingestion of fish, (b) positive reaction in skin prick test with *A. simplex* extract (see legend to Fig. 2), (c) presence of IgE to whole *Anisakis* antigens as measured by the CAP system FEIA (see legend to Fig. 2), and (d) absence of known allergy to fish or other suspected agents.

Control sera

Twenty-five sera from healthy individuals, aged 18–24 years, were obtained from a blood bank (Centro de Transfusión de Galicia, Santiago de Compostela, Spain) and used as control sera in ELISA assays.

Indirect ELISA

This assay was used to investigate the reactivity of human sera with *A. simplex* antigen bound to the ELISA plate. Briefly, the wells of polystyrene plates (Corning, New York, NY, USA) (except control wells) were coated with 1 µg of somatic antigen (SA) of *A. simplex*, obtained as previously described [14]. After washing and subsequent blocking with TRIS-buffered saline (TBS) containing 3% dry skimmed milk, the plates were incubated with human sera diluted 1:20 in TBS containing 0.2% Tween 20 (TBS-T) and 1% dry skimmed milk. Bound antibodies were detected by incubation first with peroxidase-conjugated goat antihuman Ig (polyvalent, to IgG, IgA and IgM; Sigma Chemical Co., St Louis, MO, USA; dilution 1:5000) and then with o-phenylenediamine (Sigma). The optical density (OD) was measured at 492 nm in a Biomek 2000 workstation (Beckman S.A., Madrid, Spain). For OD calculations see legends to Figs 3 and 4.

Sandwich ELISA

This procedure was used to determine the Ig isotype profile induced by the UA2- and UA3-recognized antigens [15].

First, 100 μ L of phosphate-buffered saline (PBS, pH 7.2) containing appropriate amounts of MoAb (0.8 μ g of UA2 or 1.4 μ g of UA3) was added to each well of an ELISA plate and incubated overnight at 4°C. After washing, either (a) 100 μ L of undiluted *A. simplex* SA (500 μ g total protein; for IgE determination) or (b) 100 μ L of TBS-T containing 3% skimmed dry milk (TBS-T1) and 100 μ g of *A. simplex* SA (for determination of the other Igs) or (c) 100 μ L of TBS-T1 (control without antigen) was added to each well and incubated for 1 h at 37°C. After a washing step and the addition of human serum (100 μ L of pure serum for IgE determination; 1:100 dilution in TBS-T1 for the other isotypes), the plates were incubated for 90 min at 37°C.

For determination of IgE and IgG isotypes, the plates were then washed and 100 μ L of TBS-T1 containing an appropriate dilution of the appropriate mouse antihuman MoAb labelled with fluorescein isothiocyanate (FITC) was added. The dilutions used were 1:500 for antihuman IgE (Ingenasa, Madrid, Spain) and antihuman IgG1 (Sigma), and 1:100 for antihuman IgG2, IgG3 and IgG4. After incubation for 1 h at 37°C and washing, captured Ig-FITC was detected with peroxidase-conjugated rabbit anti-FITC Ig (Dakopatts A/S, Glostrup, Denmark; dilution 1:1000 in TBS-T; 1 h incubation at 37°C).

For determination of IgA and IgM isotypes, bound serum antibodies were detected with peroxidase-conjugated polyclonal goat antihuman IgA (Sigma) or mouse antihuman IgM (Dakopatts), both pre-incubated with normal mouse serum (dilution 1:2000 in TBS-T for IgA and 1:3000 in TBS-T for IgM; 1 h incubation at 37°C). Measurement of the peroxidase activity was as described for the indirect ELISA. For OD calculations see legends to Figures 3 and 4.

Cut-off calculations

For data analysis, specific antibodies were considered to be present if the OD was more than $\bar{X} + 4$ SD, where $\bar{X}$ is the mean OD for the control sera and SD the control-group standard deviation.

Results

Anti-UA2R and anti-UA3R antibody profiles in anisakiasis sera

To compare isotype profiles induced by the antigens recognized by UA2 and UA3, we tested for UA2R- and UA3R-specific IgG1, IgG2, IgG3, IgG4, IgM and IgA antibodies in sera from patients with confirmed anisakiasis. IgE antibodies were not measured in this group because of the small amounts of serum available for study. Total levels of antibodies (IgG, IgM, IgA) against whole somatic antigens of the parasite were also determined for comparative studies.

MoAb	Isotype	Patient number						
		5	10	15	20	25	30	35
UA2	A	○	○	○	○	○	○	○
	M	○	○	○	○	○	○	○
	G1	○	○	○	○	○	○	○
	G2	○	○	○	○	○	○	○
	G3	○	○	○	○	○	○	○
	G4	○	○	○	○	○	○	○
UA3	GAM*	○	○	○	○	○	○	○
	A	○	○	○	○	○	○	○
	M	○	○	○	○	○	○	○
	G1	○	○	○	○	○	○	○
	G2	○	○	○	○	○	○	○
	G3	○	○	○	○	○	○	○

Fig. 1. Isotype profiles generated in response to the UA2R and UA3R antigens in sera from Japanese patients with confirmed anisakiasis. Black circles indicate presence of specific antibodies, and white circles absence; the corresponding raw data (ODs in ELISA) are shown in Fig. 3. Cases #8 and #17, which had no detectable IgG1 antibodies in the capture ELISA using UA3, showed CAP-determined anti-*Anisakis* IgE levels of 6.32 and 4.04 kU/L, respectively. *GAM indicates to total antibody levels (IgG, IgA and IgM) as determined in indirect ELISA using whole somatic antigens as target.

The resulting classification of sera as positive or negative for each isotype, in assays with the UA2R antigens or UA3R antigens, is shown in Figure 1. These results and those shown in Table 1 demonstrate that the antibody response to the UA2R antigens was almost exclusively IgM and IgG1 (about 50% of patients), while the response to the UA3R antigens was predominantly IgG1 (94% of sera, see Table 1). Other isotypes induced by the UA3R antigens

Table 1. Numbers of patients showing antibodies of each isotype to the antigens recognized by the monoclonal antibodies UA2 (UA2R antigens) and UA3 (UA3R antigens), considering the anisakiasis sera (Japanese patients with confirmed anisakiasis) and the allergy sera (Spanish patients with allergy to *Anisakis*). Percentages of patients testing positive are shown in brackets. The corresponding raw data (ODs in ELISA) are shown in Figs 3 and 4. Isotype profiles of individual patients are shown in Figs 1 and 2. In indirect ELISA with crude somatic antigens for determination of total antibody levels (IgG1, IgA and IgM), 59.4% of the anisakiasis patients and 90.9% of allergic patients tested positive

Isotype	UA2R antigens		UA3R antigens	
	Anisakiasis	Allergy	Anisakiasis	Allergy
A	0/22 (0)	10/19 (52.6)	1/22 (4.5)	4/19 (21.0)
M	20/37 (54.0)	18/24 (75.0)	11/37 (29.7)	28/37 (75.6)
G1	20/37 (54.0)	30/37 (81.0)	35/37 (94.5)	31/37 (83.7)
G2	1/22 (4.5)	15/35 (42.8)	0/24 (0)	12/35 (34.2)
G3	0/22 (0)	1/19 (5.2)	0/24 (0)	3/19 (15.7)
G4	0/22 (0)	1/35 (2.8)	3/24 (12.5)	19/35 (54.3)
E	ND	ND	ND	34/34 (100)

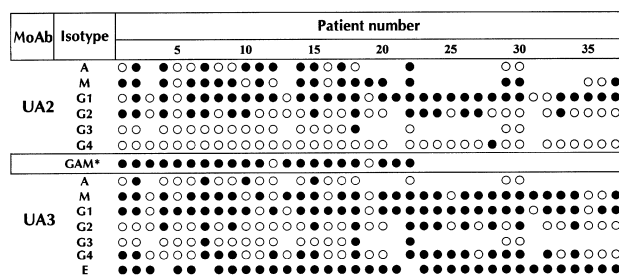


Fig. 2. Isotype profiles generated in response to the UA2R and UA3R antigens in sera from Spanish patients with allergy to *Anisakis*. Black circles indicate presence of specific antibodies, and white circles absence; the corresponding raw data (ODs in ELISA) are shown in Fig. 4. All patients were positive for IgE as measured by the Pharmacia CAP-system FEIA and prick tests. CAP values ranged from class 3 to class 6. Prick responses ranged from 3 × 4 to 7 × 9 mm diameter. *GAM indicates to total antibody levels (IgG, IgA and IgM) as determined in indirect ELISA using whole somatic antigens as target.

were also present in serum from a smaller proportion of patients (IgM in 30%, IgG4 in 12.5%). The isotype profiles obtained were independent of the time elapsed between the onset of symptoms and the extraction of the serum (< 10 h for patients 1–11; 24–72 h for patients 12–22; unknown for the remaining patients).

In addition, the results shown in Fig. 1 indicate that only one patient (#8) had IgM antibodies alone. The great majority of patients showed antibodies of more than one class.

Figure 3 shows OD values for each Ig isotype in each individual serum, in assays with the UA2R antigens (Fig. 3a) and the UA3R antigens (Fig. 3b). The total antibody determinations (IgG + IgA + IgM, as measured in indirect ELISA using crude somatic antigens) showed much lower signal-to-noise ratio (i.e. the cut-off value was high relative to positive values). Determinations in capture ELISA with the UA3R antigens showed particularly high signal-to-noise ratio.

Anti-UA2R and anti-UA3R antibody profiles in allergic sera

Compared with patients with confirmed anisakiasis, allergy patients showed more complex isotype profiles. As can be seen in Fig. 2, most of the allergy patients were positive for two or more Ig isotypes, regardless of whether UA2R or UA3R assays were considered. As for anisakiasis patients, IgA, IgG2, IgG3 and IgG4 were the least frequent isotypes. In assays with the UA2R antigens, IgG1 antibodies were present in 81% of patients, IgG2 antibodies in 43% of patients, and IgG4 antibodies in only one patient; in assays with the UA3R antigens, similar proportions of patients showed IgG1 and IgG2 antibodies (84 and 34%

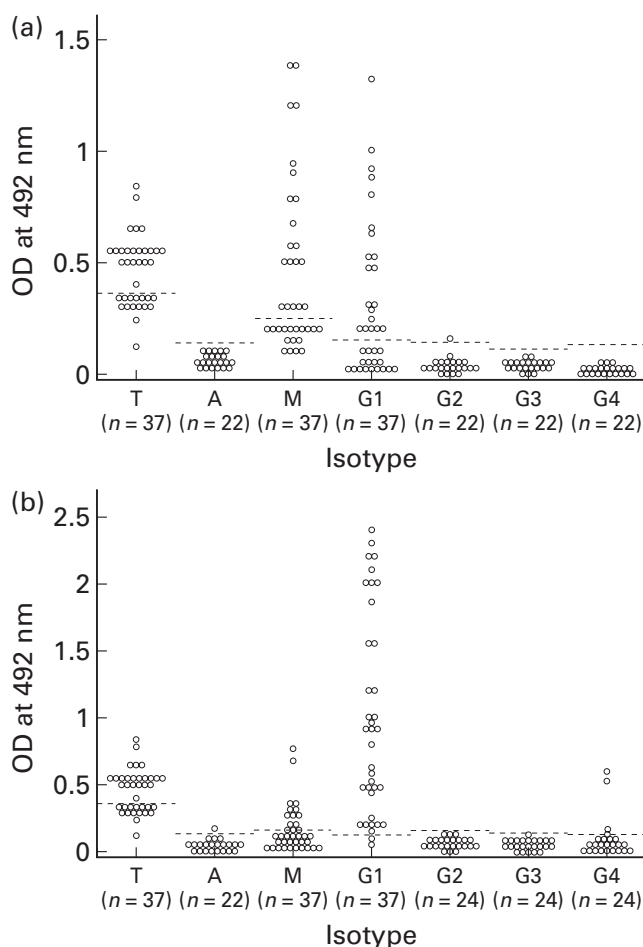


Fig. 3. UA2R-specific (a) and UA3R-specific (b) isotype levels in the Japanese anisakiasis sera; each point represents the OD value for a given patient. T = total antibodies (IgG, IgA, IgM) in indirect ELISA with whole somatic antigens of *A. simplex* as target; note the data for total antibodies shown in (a) and (b) are the same, but that the vertical scales are different. The OD value for each serum and each isotype was in all cases calculated as OD1–OD2, where OD1 is the mean of three replicate assays performed with antigen (i.e. UA2R, UA3R or whole SA), and OD2 is the mean of two replicate assays performed without antigen. Cut-off values (see text) are indicated by a dashed line.

of patients, respectively), but IgG4 antibodies were much more frequent (54% of patients) (Table 1).

It is perhaps surprising that all of these patients showed IgE antibodies to the UA3R antigens. The UA2R antigens also induce IgE antibodies (unpublished results), but the sensitivity of the corresponding ELISA is rather poor, and thus data were not included in the present study.

As for patients with confirmed anisakiasis, the capture ELISA for determination of specific isotypes showed much better signal-to-noise ratio than the indirect ELISA for determination of total Igs. The determination of IgE

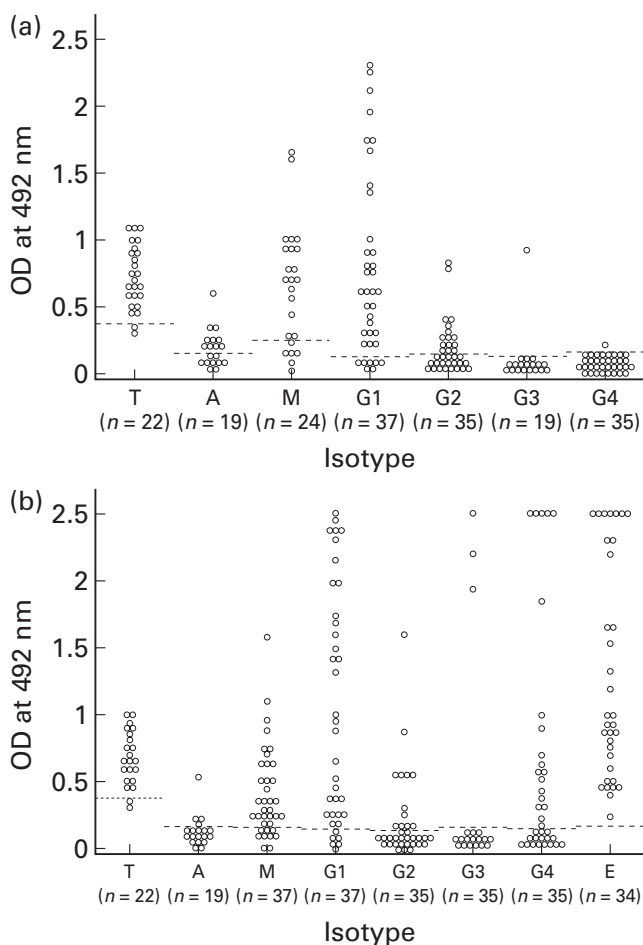


Fig. 4. UA2R-specific (a) and UA3R-specific (b) isotype levels in the Spanish allergic sera; each point represents the OD value for a given patient. T = total antibodies (IgG, IgA, IgM) in indirect ELISA with whole somatic antigens of *A. simplex* as target; note that the data for total antibodies shown in (a) and (b) are the same. The OD value for each serum and each isotype was in all cases calculated as OD1–OD2, where OD1 is the mean of three replicate assays performed with antigen (i.e. UA2R, UA3R or whole SA), and OD2 is the mean of two replicate assays performed without antigen. Cut-off values (see text) are indicated by a dashed line.

antibodies to the UA3R antigens showed a particularly strong signal.

Finally, Fig. 5 compares OD values for anti-UA3R IgE and anti-UA3R IgG4 in sera from each of the allergic patients. As can be seen, there is no relationship between the OD values obtained for the two isotypes.

Discussion

From the data obtained, it is clear that the antibody isotype responses to the *A. simplex* antigens defined by MoAbs UA2

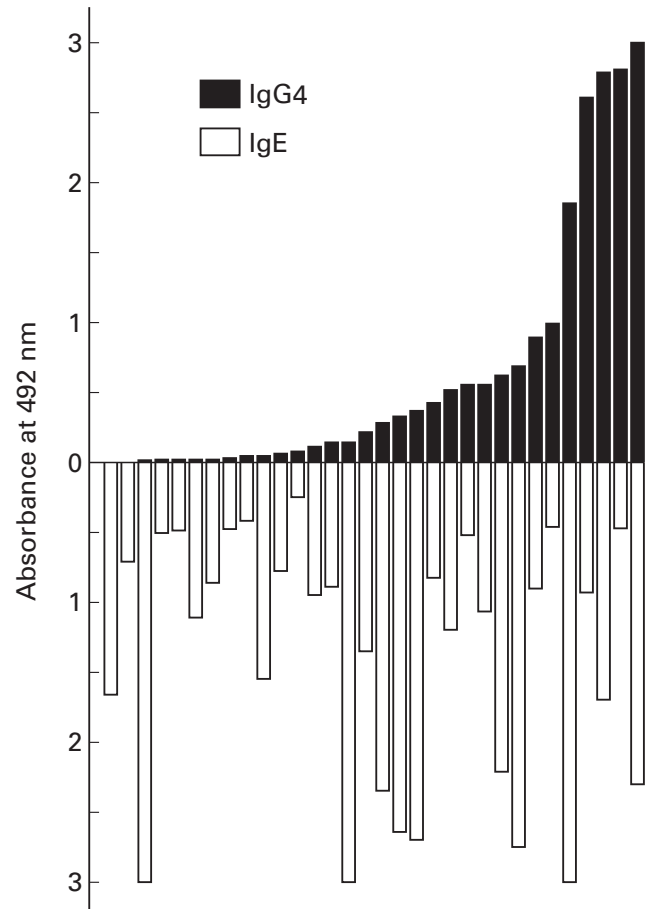


Fig. 5. Anti-UA3R IgG4 levels (black bars) and anti-UA3R IgE levels (white bars) in sera from Spanish patients with allergy to *Anisakis*. Values were obtained using undiluted serum samples for IgE, and samples diluted 1:100 for IgG4 (see Material and methods).

and UA3 are differentially regulated. Although both antigens were recognized by the majority of anisakiasis and allergy sera, as can be deduced from the IgG1 and IgM responses, the UA3R antigens also induced IgG4 antibodies in a considerable number of patients (54%). This contrasts with the UA2R antigens, which induced IgG2 in 43% of patients but IgG4 in only 2% of patients. The subclass distribution of serum IgG has been reported to vary with antigen structure, dose and route of entry, and with the genetic constitution of the host [16]. IgG1 is the prevailing isotype, sometimes in combination with IgG3, in response to thymus-dependent antigens [17]. By contrast, IgG4 is frequently associated with responses to allergens, though it can also be induced by nonallergenic antigens, particularly after repeated, long-term antigenic stimulation [18].

It is possible that the preferential induction of antibodies of the IgG2 and IgG4 subclasses may be facilitated by the

presence of immunodominant *N*-glycans on UA2R antigens, vs proteic epitopes on UA3R antigens. The presence of *N*-glycans on the UA2R antigens is suggested by the fact that binding of UA2 to its antigens is not inhibited by treatment of the latter with mild alkali, and is only partially inhibited by treatment with pronase or proteinase K [15]. Likewise, the presence of immunodominant proteic epitopes on the UA3R antigens is suggested by the fact that binding of three of our anti-*Anisakis* MoAbs (UA3, UA5 and UA6) [15] to this antigen is inhibited by proteases but not by mild alkali or PNGase F. It remains to be elucidated, however, whether individual human sera also preferentially recognize those epitopes, in the same way as mouse sera.

We detected IgE antibodies to the UA3R antigens in all allergic patients. This is surprising in view of the high M_w of these antigens (139 and 154 kDa): most allergens described to date are of low or intermediate M_w , and M_w of 70 kDa or more seem to be exceptional [19,20]. Despite this, the fact that anti-UA3R IgE was detected in all allergic patients suggests that these antigens are indeed allergenic. However, we cannot rule out the possibility that the UA3R antigens are not themselves allergenic, but rather that IgE antibodies to them are induced as a corollary of responses to other parasite allergens (as has been demonstrated for ovalbumin in rats [21]). We are currently investigating this possibility. Another possibility — that the observed IgE response was the result of polyclonal activation of B lymphocytes, with preferential production of IgE (as has been observed in response to an unidentified factor in the pseudocoelomic fluid of *A. lumbricoides* [22]) — seems less likely, since there was no correlation between total IgE and anti-UA3R IgE levels in the allergic patients.

From the point of view of diagnosis, it should be pointed out that all our allergic patients showed detectable IgG1/IgE antibodies to the UA3R antigens, indicating that they are highly immunodominant. Similarly, all but two of the anisakiasis sera showed IgG1 antibodies to the UA3R antigens. One of the negative patients (#8) had no detectable IgG1 antibodies to either the UA2R or UA3R antigens, but showed IgM antibodies to both. The other negative case (#17) showed IgG1 antibodies to the UA2R antigens but not to the UA3R antigens. One possible explanation is that this patient has only detectable antibodies of the IgE class, as was observed with some allergic patients (e.g. sera #3, #19 and #35, see Fig. 2). Alternatively, it is possible that patient #17 was sensitized to excretory–secretory antigens but not to somatic antigens (as might occur if the larva is expelled shortly after penetration into the gastrointestinal mucosa). This explanation is based on the cross-reactivity in ELISA between the somatic and excretory–secretory antigens recognized by UA2 (see above). The expulsion of *Anisakis*

larvae in vomit or faeces has been reported to be relatively frequent [14,23,24].

Our results thus suggest that the 139/154-kDa UA3R antigens are probably the most useful antigens currently available for diagnosis of both anisakiasis and *Anisakis*-induced allergy. The possibility of developing skin prick tests using UA3R proteins alone is currently under study in our laboratory.

Comparing the isotype profiles of the two patient populations (anisakiasis and allergy), it is clear that the Spanish allergic patients show higher antibody levels and greater isotype diversity than the Japanese anisakiasis patients (see Figs 1–4). This could be explained by genetic differences between European and Asian populations; however, a more probable explanation is that these differences were related either to the allergenic status of the Spanish patients or to the fact that these patients had suffered more re-immunizations and/or more prolonged contact with the parasite or its antigens than the patients from Japan (where, due to the high incidence of this parasitosis, physicians are expert in diagnosis and rapid extraction of larvae). Similar reasons may also explain the relative abundance of positive cases showing high levels of IgG4 antibodies which, as mentioned above, are frequently associated with repeated antigenic stimulation [18].

It is known that initial formation of IgE and IgG4 antibodies depends upon signals from TH2 lymphocytes and IL-4 [25], but there are also cases in which regulation of IgG4 is uncoupled from IgE [26]. Such regulation has been observed in response to a recombinant antigen in human lymphatic filariasis [27]. In the present study, we did not detect either a positive or negative correlation between anti-UA3R IgE and anti-UA3R IgG4 levels in the allergic sera. This suggests that factors other than IL-4 regulate isotype production in anisakiasis.

Finally, it has also been reported that IgA may be of value for diagnosis of anisakiasis [13]. The low levels of both anti-UA2R and anti-UA3R IgA in both patient groups argue against this possibility.

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