M.T. Ventura[1], R. Rodriguez-Perez[2], M.L. Caballero[3], M. Garcia-Alonso[2], L. Antonicelli[4], R. Asero[5]

IgE, IgG_1 and IgG_4 response to specific allergens in sensitized subjects showing different clinical reactivity to *Anisakis simplex*

[1]Interdisciplinary Department of Medicine (DIM), University of Bari Medical School, Policlinico, Bari, Italy
[2] Hospital La Paz Institute for Health Research, IdiPAZ, Madrid, Spain
[3] La Paz Hospital, Allergy Department, IdiPAZ, Madrid, Spain
[4] U.O. Allergologia, Ospedali Riuniti, Ancona, Italy
[5] Ambulatorio di Allergologia, Clinica San Carlo, Paderno Dugnano, Italy

Key words

Anisakis simplex, Food allergy; Immune response; Allergens; Immunoglobulins

Corresponding author
Riccardo Asero
Ambulatorio di Allergologia,
Clinica San Carlo
20037 Paderno Dugnano (MI), Italy
Phone: +39 02 9903 8470
Fax: +39 02 9903 8223
E-mail: r.asero@libero.it

Summary

Background. Anisakis simplex *hypersensitive subjects may be sensitized without clinical allergy, or experience acute symptoms or chronic urticaria induced by raw fish. We studied whether the 3 subgroups differ in IgE, IgG_1 or IgG_4 reactivity to specific* Anisakis simplex *allergens.* ***Methods.*** *28* Anisakis simplex*-hypersensitive adults, 11 with acute symptoms, 9 with chronic urticaria, and 8 sensitized were studied. IgE, IgG_1 and IgG_4 to rAni s 1, 5, 9 and 10 were sought by ELISA. IgE and IgG_4 to nAni s 4 were determined by WB.* ***Results.*** *IgE to Ani s 1, 4, 5, 9, and 10 were found in 8, 3, 2, 5, and 9 sera, respectively. Nine sera did not react to any allergen. IgG_1 to Ani s 1, 5, 9, and 10 were detected in 5, 16, 14, and 4 sera, respectively. Four sera did not react to any of the 4 allergens. IgG_4 to Ani s 1, 4, 5, 9, and 10 were detected in 10, 0, 2, 6 and 1 sera, respectively. Fifteen subjects did not react to any of the 5 allergens. On ELISA sensitized subjects showed lower IgE and IgG_1 levels than patients. IgG_4 levels were highest in the sensitized group. The prevalence of IgE, IgG_1 or IgG_4 reactivity to any of the studied allergens did not differ between the 3 subgroups.* ***Conclusion.*** *The clinical expression of* Anisakis simplex *sensitization does not seem to depend on IgE reactivity to a specific allergen of the parasite, nor on the presence of IgG antibodies possibly related with blocking activity.*

Introduction

Anisakiasis is a human parasitic infection of the gastrointestinal tract caused by the consumption of raw or undercooked seafood containing live larvae of the nematode *Anisakis simplex.* Many countries require all types of fish with potential risk intended for raw consumption to be previously frozen to kill parasites. However, despite the mandatory rules about the correct processing procedures required to abate the nematode present in fish for human consumption, anisakiasis occurs all over the world, due to the ingestion of the live larval stage 3 parasite found in raw or undercooked marine fish that has not undergone preventive freezing (1). Most cases are reported from Japan, but also some areas of Spain (2-4) and Italy (5) can be considered endemic for the disease. It is generally accepted that sensitization to *Anisakis simplex* follows the ingestion of raw, traditional marinated, or poorly cooked fish (6).

Three different subsets of *Anisakis simplex* hypersensitive patients can be encountered in the endemic areas: (a) patients with a history of acute systemic symptoms such as urticaria, angioedema, asthma and/or anaphylaxis upon ingestion of raw fish; (b) patients with chronic urticaria who eat marinated fish almost on a daily basis that improve after a fish-free diet (7,8); and (c) sensitized subjects who do not report any symptom despite the

frequent consumption of raw fish-based dishes. Whether such different clinical response depends on the level and type of immune response to specific *Anisakis simplex* allergens is unclear. IgG_4 antibodies have been reported to exert an IgE-blocking activity, particularly after allergen specific immunotherapy, as a result of the activities of regulatory T cells, and have been associated with the induction of immunological tolerance upon prolonged exposure to antigen (9,10), whereas IgG_1 response is the one found in normal individuals after exposure to non-self-proteins. In the present work sera from individuals belonging to the three subsets of *Anisakis simplex* hypersensitive patients have been studied, in order to detect possible differences in their humoral immune response to different *Anisakis simplex* allergens.

Patients and methods

Patients

Sera from 28 adult subjects, all sensitized to *Anisakis simplex*, as shown by unequivocally positive SPT with commercial extract of the parasite (ALK-Abellò, Madrid, Spain; 2 mg protein/ml) were used in this study. The age of study patients ranged between 18 and 77 years (mean 55; median 59), with no difference between the three subgroups. Twenty out of 28 were females; no difference in gender distribution between the subgroups was observed. Eleven patients had a history of acute symptoms (defined as the appearance of urticaria / angioedema or anaphylaxis) shortly after the ingestion or raw or marinated fish. Nine had a history of chronic urticaria (defined as the occurrence of wheals with or without angioedema on most days of the week for more than 6 weeks) that had completely and persistently resolved following the exclusion of raw or marinated fish from their diet. Eight subjects were sensitized to *Anisakis simplex* but were completely symptomless, even following the ingestion of raw or marinated fish. In order to include only patients primarily sensitized to *Anisakis simplex* and to avoid the interference of cross-reacting allergens of invertebrates, such as tropomyosin, patients positive on skin prick testing (SPT) with shrimp were excluded. Patients sensitized to fish were excluded as well. Sera from chronic urticaria patients were collected before the start of the fish-free diet. All patients underwent SPT with a large series of commercial extracts of both airborne (Pollens [grass, ragweed, mugwort, pellitory, plantain, birch, olive and cypress], moulds, mites, and cat and dog dander) and food allergens. In the acute group, 2 patients scored positive for mites, 1 for cypress pollen, and 2 for food (peanuts in both cases). In the chronic group, 3 patients scored positive for mites, 2 for pollens (both pellitory), and 3 for food (celery, maize, and egg, respectively). In the sensitized group, 3 patients scored positive for mites, 2 for pollens (both cypress and olive, one for pellitory), and 2 for food (peach and maize, respectively).

In vitro tests

Detection of specific IgE to whole *Anisakis simplex* extract
All study participants underwent the detection of IgE specific for whole *Anisakis simplex* extract by ImmunoCAP (ThermoFisher-Phadia, Uppsala, Sweden); results were expressed in kUA/L, and values > 0.35 kUA/L were considered positive.

Anisakis simplex allergens
rAni s 1 was cloned in the pPIC9 vector in the yeast *Pichia pastoris* (both kindly supplied by Dr Gabriel Salcedo from ETSIA, Madrid) and purified from the culture medium by a two steps chromatography procedure. The dialysed extracellular medium of the culture was first fractionated by cation-exchange. Subsequently, the fraction containing rAni s 1 was dialysed, freeze-dried and then separated by RP-HPLC (11). rAni s 5, rAni s 9 were obtained following the same work flow; briefly, they were cloned into the plasmid expression vector pET46 EK/LIC (Novagen, Merck KGaA, Darmstadt, Germany) that produces the protein with a histidine N-terminal tag. The resultant plasmids were transferred into E. coli BL21 Star (DE3) One Shot (Invitrogen, Carlsbad, CA, USA). Recombinant proteins were intracellularly expressed as a 6-His tagged proteins and then purified from bacterial lysate. They were purified from the soluble protein fraction using HisTrap HPcolumn (GE Health-

Table 1 *- Nature of the allergens produced and used in the study.*

Allergen tested	Source	Purified	Reference
rAni s 1	recombinant in Pichia pastoris	Cation exchange, Reversed phase HPLC	11
rAni s 5	recombinant in Escherichia coli (BL21)	Ni- Affinity cromatography	27
rAni s 9	recombinant in Escherichia coli (BL21)	Ni- Affinity cromatography	12
rAni s 10	recombinant in Escherichia coli (KRX)	Ni- Affinity cromatography	13
nAni s4	natural, extracted from A. simplex L3	Ethanol fractionation extract enriched	28

care Bio-Sciences AB) following manufacturer's specifications (12,13). Regarding rAni s 10, it was cloned into the plasmid expression vector pET46 EK/LIC, as rAni s 9 and rAni s10, but expressed in *Escherichia coli* E. coli KRX (Promega, Madison WI, USA) following manufacturer's specifications. Then it was purified from the soluble protein fraction using HisTrap HPcolumn, as for rAni 5 and rAni s9 (14) (**table 1**).

To obtain natural Ani s 4 (nAni s 4), *Anisakis simplex* L3s (1.5 g) extracted from hake muscle (*Merlucius merluccius*) were ground in PBS (5 ml) with a mortar and pestle and the mixture was centrifuged at 4,000 g for 15 min. The supernatant obtained was mixed with 5 ml of ethanol, incubated for 30 min on ice and centrifuged in the same conditions. Again, the supernatant was mixed with ethanol to increase the ethanol concentration to 66%, incubated for 30 min on ice and centrifuged. The supernatant was discarded and the pellet was re-suspended in 500 µl of dH_2O (15) (**table 1**).

Detection of specific IgE, IgG_1, and IgG_4 to rAni s 1, rAni s 5, rAni s 9 and rAni s 10 by ELISA

Polystirene 96-well plates (Costar 3590, Corning, NY, USA) were coated for 2 h at 37 ºC with 100 ml of recombinant allergen at 10 mg/ml in carbonate buffer pH 9.6. The coated wells were blocked with 1% BSA in PBS for 30 min at 37 ºC, and then incubated overnight at room temperature with 100 ml of patient's serum (1/4 dilution in 1% BSA, 0.05% Tween-20, in PBS). After washing with 0.05% Tween-20 in PBS, wells were incubated for 1 h at room temperature with peroxidase-labelled anti human IgE (SouthernBiotech, Birmingham, AL, USA; 1/2000 dilution), or peroxidase-labelled anti human IgG_1 (SouthernBiotech; 1/8000 dilution), or peroxidase-labelled anti human IgG_4 (SouthernBiotech; 1/1000 dilution). Plates were washed again and, and then developed with 100 ml of TMB-turbo ELISA substrate (Thermo Scientific, Rockford, IL, USA). The reaction was stopped after 30 min with 10 ml of 2N H_2SO_4, and the optical density (OD) was measured at 450 nm. Assays were performed in duplicate. Blocking buffer was used as negative control. For data analysis, sera were considered positive if OD was more than arithmetic mean plus 3SD (standard deviation), calculated from the results of 25 control sera from non-atopic individuals tested for IgE, IgG_1 and IgG_4. The cut-off values for IgE, IgG_1 and IgG_4 determinations were 0.14 for rAni s 1 and 0.12 for rAni s 5, rAni s 9 and rAni s 10.

Detection of IgE and IgG_4 to nAni s 4

Specific IgE and IgG_4 against nAni s 4 were determined by western-blotting with an extract enriched in this allergen. Extract containing nAni s 4 (5 µg), was fractionated by 16% SDS-PAGE and then separated proteins were transferred onto nitrocellulose membranes by diffusion (Nitro-Pure supported, 0.45 µm, GE Osmonics Labstore, Minnetonka, MN, USA) to perform a western-blotting.

The membranes were washed and blocked with 3% Nonidet NP-40 (Amresco, Solon, OH, USA) in PBS for 30 minutes. After that, they were incubated overnight with individual sera from the patients (1:10 dilution). Specific IgE or specific IgG_4 detection was carried out with monoclonal anti-human-IgE (1:1000) (Ingenasa, Madrid, Spain) or monoclonal anti-human IgG_4 (1:000) (Sigma-Aldrich, St Louis, MO, USA), respectively. Subsequently, membranes were incubated with alkaline phosphatase-labelled goat anti-mouse antibody (1:2500) (Sigma-Aldrich, St Louis, MO, USA). Finally, the signal was visualized with the alkaline phosphatase 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/4-nitroblue tetrazolium (NBT) system (Amresco) for 30 min.

Statistics

Qualitative variables have been summarized with their relative frequency. Pearson $\chi 2$ test was applied for the bivariate comparison of proportions. Likelihood test of independence was used when the application conditions of the $\chi 2$ test were not met. The Kruskal-Wallis nonparametric test was used for the comparison of quantitative variables in independent samples.

Probability values < 0.05 were considered statistically significant. Data analysis was performed using the software SPSS 15.0 (SPSS Inc., Chicago, IL).

Results

Results are summarized in **table 2**. All study participants but 2 (both with a history of chronic urticaria) showed circulating IgE to whole *Anisakis simplex* extract tested by ImmunoCAP; one of those two negative subjects reacted to Ani s 10 on ELISA. The three subgroups of patients studied (sensitized, acute and chronic) did not show any statistically significant difference in terms of *Anisakis*-specific IgE levels tested by ImmunoCAP. The two subjects showing the highest levels of IgE to *Anisakis simplex* extract belonged to the subgroup showing symptomless sensitization.

IgE specific for rAni s 1, nAni s 4, rAni s 5, rAni s 9, and rAni s 10 were found in 8, 3, 2, 5, and 9 sera, respectively. Nine sera did not show IgE reactivity to any of the studied allergen components.

IgG_1 to rAni s 1, rAni s 5, rAni s 9, and rAni s 10 were detected in sera from 5, 16, 14, and 4 subjects, respectively. Sera from 4 subjects did not show IgG_1 reactivity to any of the 4 allergens. IgG_1 to rAni s 5 and rAni s 9 were frequently detected in the absence of the corresponding IgE response.

IgG_4 to rAni s 1, nAni s 4, rAni s 5, rAni s 9, and rAni s 10 were detected in 10, 0, 2, 6, and 1 sera respectively. Sera from 15 subjects did not show IgG_4 reactivity to any of the 5 allergens

Table 2 - *Detection of IgE, IgG_1, and IgG_4 against different Anisakis simplex allergen proteins. Positive values are highlighted in bold.*

					rAni s 1			nAni s 4		rAni s 5			rAni s 9			rAni s 10		
	Code	**sex**	**age**	**CAP**	**IgE**	**IgG1**	**IgG4**	**IgE**	**IgG4**	**IgE**	**IgG1**	**IgG4**	**IgE**	**IgG1**	**IgG4**	**IgE**	**IgG1**	**IgG4**
	A1	M	77	75.00	**1.13**	**0.88**	**0.93**	**+**	-	**0.43**	**0.14**	0.03	**1.15**	**1.60**	**0.86**	0.04	0.02	0.03
	A2	F	65	3.45	0.00	0.13	0.13	-	-	0.02	0.03	0.01	0.01	0.04	0.04	0.00	0.07	0.01
	A3	F	37	16.0	0.02	0.06	0.02	-	-	0.06	0.09	0.00	**0.91**	**1.65**	**0.74**	0.03	0.06	0.01
	A4	F	49	3.2	0.01	0.11	0.10	-	-	0.05	**0.44**	0.00	0.01	0.10	0.06	0.00	0.09	0.02
	A5	F	55	1.01	0.02	0.05	0.02	-	-	0.01	0.08	0.07	0.00	**0.41**	0.08	0.04	0.02	0.05
Acute	A6	F	75	1.12	0.02	0.13	**0.72**	-	-	0.00	**0.67**	0.05	0.00	0.12	0.05	0.06	0.02	0.01
	A7	F	60	2.65	0.01	0.08	0.10	-	-	0.01	**0.42**	0.04	0.00	0.08	0.10	**0.14**	0.06	0.10
	A8	F	54	15.60	**1.08**	0.13	**1.08**	-	-	**0.27**	**0.74**	**0.91**	0.00	**0.19**	0.05	0.03	0.03	0.03
	A9	F	24	5.60	0.00	**0.27**	0.03	-	-	0.01	**1.34**	0.02	0.00	0.10	0.02	**0.17**	0.06	0.00
	A10	M	18	0.36	0.00	0.08	0.05	-	-	0.00	**0.21**	0.02	0.00	0.10	0.04	**0.20**	0.05	0.00
	A11	M	26	0.36	0.00	**0.17**	0.04	-	-	0.01	**0.34**	0.02	0.00	**0.51**	0.04	0.00	0.09	0.01
	S1	F	75	40.80	0.00	0.05	0.13	**+**	-	0.01	**0.23**	0.01	0.00	**0.28**	0.02	0.02	0.06	0.08
	S2	F	72	0.36	0.00	0.01	0.05	-	-	0.00	0.10	0.03	0.00	**0.21**	0.03	0.02	0.03	0.02
	S3	F	62	19.40	**0.95**	0.06	**1.07**	**+**	-	0.01	0.05	0.02	**0.31**	**0.45**	**0.61**	0.00	**0.16**	0.02
Sensitized	S4	M	49	17.30	0.00	0.09	**1.12**	-	-	0.09	**0.33**	**0.68**	0.00	0.05	0.07	**0.15**	**0.14**	**0.37**
	S5	M	22	3.30	0.00	**0.19**	0.02	-	-	0.00	0.04	0.06	0.00	0.04	0.03	**0.14**	0.08	0.10
	S6	M	62	93.00	0.05	0.08	0.07	-	-	0.00	0.06	0.08	**0.33**	**1.32**	**0.84**	0.08	0.04	0.01
	S7	F	53	90.00	**0.55**	**0.30**	**0.86**	-	-	0.00	0.11	0.05	0.00	0.03	**0.25**	0.09	0.08	0.10
	S8	F	71	14.00	0.00	0.03	**0.17**	-	-	0.01	0.10	0.07	0.00	0.03	0.08	**0.13**	0.01	0.10
	C1	F	72	13.14	**0.95**	0.09	**1.04**	-	-	0.00	**0.36**	**0.13**	0.00	0.05	0.10	0.09	0.08	0.05
	C2	F	76	2.96	**0.20**	0.07	**1.08**	-	-	0.00	0.08	0.02	0.00	0.02	0.02	**0.17**	0.03	0.01
	C3	F	30	0.00	0.01	0.07	0.04	-	-	0.00	0.04	0.02	0.00	**0.58**	0.04	**0.17**	0.04	0.00
Chronic	C4	F	44	0.73	0.01	0.08	0.05	-	-	0.01	**0.50**	0.03	0.00	**0.71**	0.06	**0.14**	0.02	0.00
	C5	F	67	8.74	**0.57**	0.12	0.11	-	-	0.01	**0.78**	0.01	**0.22**	**0.39**	0.10	0.07	**0.37**	0.01
	C6	F	65	0.75	0.01	0.08	0.09	-	-	0.01	**0.36**	0.07	0.00	**0.70**	**0.26**	0.07	0.04	0.08
	C7	F	75	3.41	0.08	0.08	**0.15**	-	-	0.01	**0.39**	0.05	0.00	0.03	0.03	0.04	0.03	0.02
	C8	M	57	40.50	**0.77**	0.05	0.02	-	-	0.02	**0.19**	0.11	0.00	0.09	0.04	0.08	**0.16**	0.03
	C9	M	43	0.00	0.01	0.11	0.00	-	-	0.00	0.07	0.00	0.00	**0.18**	0.01	0.00	0.01	0.01

A1-11: patients with a history of acute reactions. S1-8: Subjects sensitized to *Anisakis simplex* without any clinical symptom. C1-9: patients with *Anisakis simplex*-induced chronic urticaria.
CAP values to whole *Anisakis simplex* extract are expressed as kUA/L (n.v. < 0.35). The cut-off values were 0.14 for rAni s 1 and 0.12 for rAni s 5, rAni s 9 and rAni s 10.

studied. An IgG_4 response in the absence of the corresponding IgE response was found in 7 instances (4 rAni s 1, 1 rAni s 5, 2 rAni s 9) (**table 3**).

We compared the specific IgE values tested by ImmunoCAP and by ELISA with recombinant allergens. The main differences were observed in the sensitized group, since the CAP values were much higher (and dispersed) than in the acute and chronic patients. On the contrary, when we analysed the specific IgE levels considering at least the highest positive of the four allergens tested by ELISA, we found that specific IgE levels were lower among the sensitized patients than among the acute or the chronic patients (**figure 1**, panels A and B). Nonetheless , a correlation coefficient after Pearson calculated for ImmunoCAP values and the sum of the ELISA results for each individual sample showed a weak, albeit statistically significant, correlation ($r= 0.4$; $p< 0.05$).

We analyzed the specific IgE levels considering at least one positive of the four allergens tested by ELISA, and we found that specific IgE levels were lower among the sensitized patients than among the acute or the chronic patients. A similar pattern was observed for IgG_1, with the highest levels observed among the acute patients. Regarding IgG_4 levels, the tendency was the opposite, being the highest levels present in the sensitized group and the wider range of values among the chronic patients (Figure 1, panels B to D). However these differences did not reach the significance level.

The 3 groups did not show any statistically significant difference in the prevalence of either IgE or IgG reactivity to any of the studied allergens.

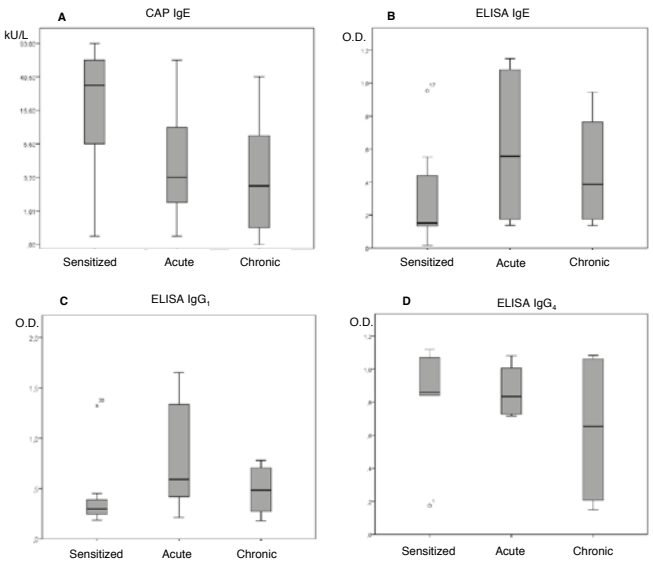
Table 3 *- Allergen detection comparison between Anisakis simplex-induced acute or chronic pathology and Anisakis simplex sensitized individuals.*

		Sensitized n = 8	Acute n = 11	Chronic n = 9	p value
rAni s 1	IgE	2 (25%)	2 (18%)	4 (44%)	0.425
	IgG_1	2 (25%)	3 (27%)	0 (0%)	0.111
	IgG_4	4 (50%)	3 (27%)	3 (33%)	0.589
nAni s 4	IgE	2 (25%)	1 (9%)	0 (0%)	0.186
	IgG_4	0 (0%)	0 (0%)	0 (0%)	--
rAni s 5	IgE	0 (0%)	2 (18%)	0 (0%)	0.137
	IgG_1	2 (25%)	8 (72%)	6 (66%)	0.086
	IgG_4	1 (12%)	1 (89%)	1 (11%)	0.971
rAni s 9	IgE	2 (25%)	2 (18%)	1 (11%)	0.752
	IgG_1	4 (50%)	5 (45%)	5 (55%)	0.904
	IgG_4	3 (37%)	2 (18%)	1 (11%)	0.406
rAni s 10	IgE	3 (37%)	3 (27%)	3 (33%)	0.891
	IgG_1	2 (25%)	0 (0%)	2 (22%)	0.109
	IgG_4	1 (12%)	0 (0%)	0 (0%)	0.273
At least one positive	IgE	7 (87%)	6 (54%)	6 (66%)	0.284
	IgG_1	7 (87%)	10 (90%)	8 (88%)	0.971
	IgG_4	5 (62%)	4 (36%)	4 (44%)	0.521

Discussion

IgE plays a fundamental role in allergic diseases since it is responsible of allergic symptoms, whereas IgG_4 immunoglobulin has been associated with the suppression of IgE-dependent immediate hypersensitivity reactions which occur, for instance,

Figure 1 *- Comparison between Ig levels to recombinant Anisakis simplex allergens in sensitized, acute and chronic patients. A: sIgE CAP assay, B: sIgE ELISA with recombinant Anisakis simplex allergens assay, C: $sIgG_1$ ELISA with recombinant Anisakis simplex allergens assay and D: $sIgG_4$ ELISA with recombinant Anisakis simplex allergens assay. The ELISA value chosen for each patient corresponds to the highest positive of the allergens tested (rAni s 1, rAni s 5, rAni s 9 or rAni s 10).*



during helminthic infections. In this sense, it has been proposed that the regulation of isotype switching is an important checkpoint in the development of clinical allergy versus tolerance, and that IgG_4 may act by blocking IgE access to the antigen (16). According to this theory, our initial hypothesis was that both patients with chronic urticaria and sensitized but asymptomatic subjects might show a higher prevalence of specific IgG_4 response and lower levels of specific IgE than patients with a history of acute symptoms following the ingestion of raw fish. In this sense, Daschner et al. (8) found that patients with both chronic urticaria and *Anisakis simplex* sensitization show a clear positive response to a two-month fish-free diet particularly if they show specific IgG_4 levels to the parasite. The same group detected a higher IgG avidity among patients with gastro-allergic anisakiasis (GAA, corresponding to our "acute" subset) than in patients with *Anisakis simplex* sensitization-associated chronic urticaria, whereas the former subgroup showed a statistically non-significant trend to a lower IgE avidity (17). Further, they found higher IgG_4 and IgE levels to *Anisakis simplex* crude extract and higher IgG_4 levels to both Ani s 1 and Ani s 7 in patients with GAA (18). In the present study we found that among the three subsets studied (sensitized, acute, or chronic)

sensitized subjects showed the lowest specific IgE levels tested by ELISA and the highest levels of specific IgG_4; chronic patients showed the widest range of values. We observed a similar pattern when we compared the specific IgE and specific IgG_1 responses, with the highest levels observed among acute patients (**figure 1**) although the tendency observed did not reach the significance level, maybe due the low number of patients studied.

Another interesting finding was the different distribution of specific IgE determined by ImmunoCAP or by ELISA among the three subsets of patients. On ImmunoCAP, the highest values were found among sensitized, asymptomatic subjects, which may seem illogical from an immunological point of view. A possible explanation might be that ImmunoCAP reflects the presence of IgE to *Anisakis simplex* proteins that, although able to fix IgE, are less active in triggering allergic symptoms; another explanation might be that, since the assay is performed with a whole extract of the parasite, it reflects sensitizations that are not primarily directed to *Anisakis simplex*. In this sense, cross-reactions with crustaceans (19), mites (20) and other nematodes like Ascaris (21) have been reported. Another fact to take into account is the detection of carbohydrate-type determinants of parasite antigens; in a group of sensitized Spanish patients, sIgE CAP values dropped by 27.44% on average (range 0-48.62%) after CAP inhibition assay with bromelin as source of CCDs (22). This means that CCDs may interfere with accuracy when detecting clinically relevant sIgE, and to make things even worse, the magnitude of the interference may differ from one serum to another. This could explain the more dispersed data of sIgE CAP when compared with ELISA performed with recombinant allergens. Furthermore, the higher specificity for the diagnosis of rAni s 1, rAni s 5, rAni s 9 and rAni s 10 compared with ImmunoCAP has been demonstrated in a Spanish population (22), where this set of allergens diagnoses up to 99.99% of the allergic population. In other populations some other allergens might be included in order to cover the whole population for diagnostic purposes. These observations are in keeping with a very recent study of respiratory allergy showing that the levels of IgE specific for extracts were higher than the levels of IgE specific for the corresponding components (23)

Although a statistically significant correlation between ImmunoCAP and the sum of ELISA results was found, the ELISA with recombinant allergens seems more specific than the ImmunoCAP. This is consistent with the results of our previous study (24) in which sensitized subjects showed lower IgE levels than patients. If specific IgE were really low among sensitized (clinically asymptomatic) subjects, one might speculate that the IgE response to *Anisakis simplex* is normal or even protective, as has been suggested for honeybee venom (25). The repeated exposition to the parasite would lead to an increase in both IgE and IgG_4 without a clear predominance between them; this might not be necessarily the expression of a severe disease, but rather of a long-term interaction between host and parasite with the occurrence of an unstable balance (26,27) whose clinical expression could be represented by patients with *Anisakis simplex* sensitization-associated chronic urticaria. The altered intestinal permeability and its reversibility following a fish-free diet (28,29) might be the expression of this instable equilibrium in the intestinal tract. In this scenario, the more severe allergic / anaphylactic reactions might reflect an interaction between the allergen and specific IgE that occurs in patients in whom conditions triggering anaphylaxis coexist (30); obviously, such conditions could not be predicted on the basis of either specific IgE (31) or IgG_4 (32) levels. In fact, in the three subsets studied here specific IgE and IgG_4 were largely overlapping and do not allow a clear distinction between the different clinical conditions. This scenario does not seem to fit with the protective role of specific IgG_4 levels such as that observed in beekeepers (25); however, in that model the dose of allergen as well as the timing of stings are known, whereas both these conditions are largely unknown in the long-term interaction between the immune system and *Anisakis simplex*.

Of course, we cannot exclude at all that the results of the present study are due to the low numbers of subjects studied within each group; however, the two highest levels of *Anisakis simplex* IgE were detected in asymptomatic but sensitized subjects. In conclusion, these data suggest that the clinical expression of *Anisakis simplex* sensitization depends on factors other than the mere presence of specific IgE. Further, in keeping with the model of food allergies (33), our observations suggest that IgG do not seem to exert any "blocking" or preventive effect in subjects sensitized to the parasite.

Acknowledgments

The study was founded by by Fondo de Investigacion Sanitario (FIS) from Ministry of Economia y Competitividad (grant No. PI12/00581). Dra. Rosa Rodriguez-Perez is supported by "Miguel Servet 2" Program from FIS with FEDER funds.

We are indebted to Dr. Carmen de Burgos from Epidemiology Unit of Hospital Carlos III for the statistical analysis and the technician Beatriz Sanz Minguela for her technical assistance.

References

1. Ishikura H, Kikuchi K, Nagasawa K, Ooiwa T, Takamiya H, Sato N, Sugane K. Anisakidae and Anisakidosis. Prog Clin Parasitol. 1993;3:43-102.
2. Audicana MT, Ansotegui IJ, Fernández de Corres L, Kennedy MW. *Anisakis simplex*: dangerous- dead and alive? Trends in Parasitology. 2002;18:20-5.
3. Daschner A, Pascual CY. *Anisakis simplex*: sensitization and clinical allergy. Curr Opin Allergy Clin Immunol. 2005;5:281-5.

4. Moneo I, Caballero ML, Rodriguez-Perez R, Rodriguez-Mahillo A, Gonzalez-Muñoz M. Sensitization to the fish parasite *Anisakis simplex*: clinical and laboratory aspects. Parasitol Res. 2007;101:1051-5.
5. AAITO-IFIACI Anisakis Consortium. Anisakis hypersensitivity in Italy: prevalence and clinical features: a multicenter study. Allergy. 2011;66:1563-9.
6. Fernandez de Corres L, Del Pozo MD, Aizpuru F. Prevalence of sensitization to *Anisakis simplex* in three Spanish areas, regarding the rates of fish consumption. Relevance of *Anisakis simplex* allergy. Alergol Inmunol Clin. 2001;16:337-46.
7. Ventura MT, Napolitano S, Menga R, Cecere R, Asero R. *Anisakis simplex* hypersensitivity is associated with chronic urticaria in endemic areas. Int Arch Allergy Immunol. 2013;160:297-300.
8. Daschner A, Vega de la Osada F, Pascual CY. Allergy and parasites re-evaluated: wide-scale induction of chronic urticaria by the ubiquitous fish-nematode Anisakis simplex in an endemic region. Allergol Immunopathol. 2005;33:31-7.
9. Flicker S, Valenta R. Renaissance of the blocking antibody concept in type I allergy. Int Arch Allergy Immunol. 2003;132:13-24.
10. Satoguina J, Weyand E, Larbi J, Hoerauf A. T regulatory-1 cells induce IgG_4 production by B cells: role of IL-10. J Immunol. 2005;174:4718-26.
11. Rodriguez-Perez R, Caballero ML, Gonzalez-Munoz M, Rodriguez-Mahillo A, Moneo I. Cloning and expression of a biologically active *Anisakis simplex* allergen Ani s 1 in the yeast *Pichia pastoris*. Mol Biochem Parasitol. 2007;154:115-8.
12. Rodriguez-Perez R, Moneo I, Rodriguez-Mahillo A, Caballero M L. Cloning and expression of Ani s 9, a new *Anisakis simplex* allergen. Mol Biochem Parasitol. 2008;159:92-7.
13. García-Mayoral MF, Treviño MA, Pérez-Piñar T, Caballero ML, Knaute T, Umpierrez A, Bruix M, Rodríguez-Pérez R. Relationships between IgE/IgG4 Epitopes, Structure and Function in Anisakis simplex Ani s 5, a Member of the SXP/RAL-2 Protein Family. PLoS Negl Trop Dis. 2014;8:e2735.
14. Caballero ML, Umpierrez A, Moneo I, Rodriguez-Perez R. Ani s 10, a new *Anisakis simplex* allergen: cloning and heterologous expression. Parasitol Int. 2011;60:209-12.
15. Moneo I, Caballero ML, Gonzalez-Munoz M, Rodriguez-Mahillo AI, Rodriguez-Perez R, Silva A. Isolation of a heat-resistant allergen from the fish parasite Anisakis simplex. Parasitol Res. 2005;96:285-9.
16. Hussain R, Poindexter RW, Ottesen EA. Control of allergic reactivity in human filariasis. Predominant localization of blocking antibody to the IgG_4 subclass. J Immunol 1992; 141:119-129.
17. Cuellar C, Valls A, de Frutos C, Rodero M, Daschner A. Avidity studies in *Anisakis simplex*-associated allergic diseases. J Allergy (Cairo). 2013;2013:106781.
18. Daschner A, Fernandez-Figares V, Rodero M, Valls A, de Frutos C, Ubeira FM, Cuellar C. Specific IgG_4: possible role in the pathogenesis and a new marker in the diagnosis of Anisakis-associated allergic disease. Scand J Immunol. 2014;79:120-6.
19. Pascual CY, Crespo JF, San Martin S, Ornia N, Ortega N, Caballero T, Muñoz-Pereira M, Martin-Esteban M: Cross-reactivity between IgE-binding proteins from Anisakis, German cockroach, and chironomids. Allergy. 1997;52:514-20.
20. Johansson E, Aponno M, Lundberg M, van Hage-Hamsten M: Allergenic cross-reactivity between the nematode Anisakis simplex and the dust mites Acarus siro, Lepidoglyphus destructor, Tyrophagus putrescentiae and Dermatophagoides pteronyssinus. Allergy. 2001;56:660-6.
21. Carballeda-Santiago N, Rodríguez-Mahillo AI, Puente S, Gutiérrez MT, Moneo I, González-Muñoz M. Anisakis/Ascaris IgE ratio improves specificity for the diagnosis of Anisakis simplex sensitization in travellers and immigrants. Acta Trop. 2014;138:1-4.
22. Caballero ML, Umpierrez A, Perez-Piñar T, Moneo I, de Burgos C, Asturias JA, Rodríguez-Pérez R. Anisakis simplex recombinant allergens increase diagnosis specificity preserving high sensitivity. Int Arch Allergy Immunol. 2012;158:232-40.
23. Patelis A, Gunnbjornsdottir M, Alving K, Borres MP, Hogman M, Janson C, Malinovschi A. Allergen extract vs component sensitization and airway inflammation, responsiveness and new onset respiratory disease. Clin Exp Allergy. 2016;46:730-40.
24. Caballero ML, Asero R, Antonicelli L, Kamberi E, Colangelo C, Fazii P, de Burgos C, Rodriguez-Perez R. Anisakis allergy component-resolved diagnosis: clinical and immunologic differences between patients from Italy and Spain. Int Arch Allergy Immunol. 2013;162:39-44.
25. Marichal T, Starkl P, Reber LL, Kalesnikoff J, Oettgen HC, Tsai M, Metz M, Galli SJ. A beneficial role for immunoglobulin E in host defense against honeybee venom. Immunity. 2013;39:963-75.
26. Hussaarts L, van der Vlugt LE, Yazdanbakhsh M, Smits HH. Regulatory B-cell induction by helminths: implications for allergic disease. J Allergy Clin Immunol. 2011;128:733-9.
27. Yazdanbakhsh M, Kremsner PG, van Ree R. Allergy, parasites, and the hygiene hypothesis. Science. 2002;296:490-4.
28. Ventura MT, Polimeno L, Amoruso AC, Gatti F, Annoscia E, Marinaro M, Di Leo E, Matino MG, Buquicchio R, Bonini S, Tursi A, Francavilla A. Intestinal permeability in patients with adverse reactions to food. Dig Liver Dis. 2006;38:732-6.
29. Polimeno L, Loiacono M, Pesetti B, Mallamaci R, Mastrodonato M, Azzarone A, Annoscia E, Gatti F, Amoruso A, Ventura MT. Anisakiasis, an Underestimated Infection: Effect on Intestinal Permeability of *Anisakis simplex*-Sensitized Patients. Foodborne Pathog Dis. 2010;7:809-14.
30. Vadas P, Perelman B, Liss G. Platelet-activating factor, histamine, and tryptase levels in human anaphylaxis. J Allergy Clin Immunol. 2013;131:144-9.
31. Pravettoni V, Piantanida M, Primavesi L, Forti S, Pastorello EA. Basal platelet-activating factor acetylhydrolase: Prognostic marker of severe Hymenoptera venom anaphylaxis. J Allergy Clin Immunol. 2014;133:1218-20.
32. Meiler F, Zumkehr J, Klunker S, Rückert B, Akdis CA, Akdis M. In vivo switch to IL-10-secreting T regulatory cells in high dose allergen exposure. J Exp Med. 2008;205:2887-98.
33. Stapel SO, Asero R, Ballmer-Weber BK, Knol EF, Strobel S, Vieths S, Kleine-Tebbe J. Testing for IgG_4 against food is not recommended as a diagnostic tool: EAACI task force report. Allergy 2008;63:793-6.