

Gastro-allergic anisakiasis as a consequence of simultaneous primary and secondary immune response

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SUMMARY

Gastro-allergic anisakiasis has been reported as an entity in which an acute parasitism by Anisakis simplex is accompanied by an immunoglobulin (Ig)E-mediated systemic allergic reaction. Serum samples were obtained from 24 patients within 24 h after the onset of symptoms (day 0) and after 1 month (day 30) and in 13 patients after 6 months. Total IgE was assessed by the Imx method. Specific IgE was assessed by CAP-FEIA. Specific IgM, IgG, IgG4 and IgA antibodies were assessed by enzyme-linked immunosorbent assay against crude extract (CE) and excretory–secretory products (ESP). IgE immunoblotting (IB) was directed against CE or ESP (day 0 and day 30). We found a rise of total IgE, specific IgE, number of bands in IgE-IB, IgG and IgG4 between day 0 and day 30 with a fall to near basal levels after 6 months. IgM levels were highest at day 0, falling over the next 6 months and IgA levels remained almost unchanged. Correlation studies revealed a parallel stimulation of nearly all Ig isotypes, except IgM anti-ESP, whose antibody levels correlated negatively with specific IgG levels. We found an extension of the IgE antibody repertoire in IB. We conclude that the allergic IgE-mediated reaction in the course of gastro-allergic anisakiasis involves a parallel secondary Th2 type memory response and a primary immunologic stimulation of both Th2 and Th1 lymphocyte subsets against previously unrecognized antigens.

Keywords *Anisakis simplex, nematode, immunoparasitology, allergy, immunoglobulin, polyclonal stimulation*

INTRODUCTION

Gastric anisakiasis is caused by the third stage larva of the nematode *Anisakis simplex* (*A.s.*). It has been known since the early 1960s and has been reported from all over the world. The larva is acquired by eating raw or undercooked fish.

In recent years, the allergenic potential of *A.s.* has been described and numerous articles have been published in this respect. The involvement of an IgE-mediated response has been proven by means of skin prick tests (SPTs) and specific immunoglobulin (Ig)E against this nematode (1–3). It is still to be proven if allergic symptoms also appear without an acute parasitism. In this respect, we described gastro-allergic anisakiasis as an acute parasitism with hypersensitivity symptoms appearing after ingestion of raw or undercooked fish, sometimes many hours after the intake of the implicated fish (4–6). Patients with previous gastro-allergic anisakiasis do not show new acute hypersensitivity symptoms if they eat well-cooked or deep-frozen fish (unpublished observations) and we also demonstrated, in these patients, oral tolerance of deep-frozen, noninfective *A.s.* larvae (7). These results led to the hypothesis, that the clinically relevant allergens of *A.s.* could be excretory–secretory products (ESP) released only by the live larva in the context of an acute parasitism (6).

In acute gastro-allergic anisakiasis, the precise moment of infection is not difficult to determine, in contrast to the chronic forms of most parasitic diseases. This entity can be considered as a ‘natural experiment’ as the moment of entry and contact of the nematode with the patient can be determined by the patient’s clinical history.

We have previously documented an important rise of total and specific IgE against *A.s.* 1 month after acute gastro-allergic anisakiasis and this was considered as a reaction induced by the live parasitic larva in the context of a polyclonal immune stimulation (8). This is a typical feature of parasitic disease but, to date, documentation of a rise of

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IgE after acute parasitism has only been shown in animal experiments.

The objective of this study was to observe the kinetics of specific anti-*Anisakis* antibodies of different immunoglobulin isotypes. For this purpose, over a period of 6 months, we examined specific antibodies against crude extract (CE) and ESP of *A.s.* and, by IgE-immunoblotting (IB), possible changes in the antibody repertoire of the selected patients.

MATERIALS AND METHODS

Patient's selection and study design

A total of 24 patients were selected according to the following criteria indicating likely gastro-allergic anisakiasis (6,8): acute allergic type I hypersensitivity symptoms such as urticaria, angioedema, bronchospasm in the 24-h period after ingestion of raw or undercooked fish; exclusion of other suspected agents like other food, drugs and infections; positive SPTs and specific IgE against *A.s.* and negative SPT against implicated fish. Mean (SD) patient age was 46 (15.8) years.

In all patients, a serum sample was taken within 24 h of the onset of symptoms (day 0) and after 1 month (day 30). In 13 of 24 patients, a serum sample could be obtained after 6 months. (In only eight of these 13 patients could a whole immunological evaluation be performed.) Sera were stored until laboratory analysis at -70°C .

Data were obtained with respect to age and previous episodes of allergic type I hypersensitivity symptoms with fish.

Antigen

Anisakis simplex crude extract was prepared from the third larval stage (L3). The larvae were obtained from hake or blue whiting flesh and viscera provided by a local fish market. After identification, the larvae were ground in liquid nitrogen for 5 min with a pestle. The powdered material was then resuspended with phosphate-buffered saline (PBS) (0.01 M sodium phosphate, 0.15 M NaCl). The mixture was stirred for 4 h and centrifuged at 15 000 *g* for 30 min at 4°C . The supernatant was dialysed (Spectrapor, Spectrum Medical Industries, Los Angeles, CA, USA, molecular weight cut-off 3.5 kDa) at 4°C for 24 h against 10 mM glycine, centrifuged at 30 000 *g* for 30 min at 4°C , and passed through a 0.45- μm Millipore filter (Millipore, Bedford, MA, USA). Finally, the material was lyophilized.

To obtain ESP, viable *A.s.* L3 were selected and cultured for 1 h in NaCl (0.85%) containing gentamicin (4 g/l). Then larvae were placed in Minimum Essential Eagle Medium with Earle's salts (ICN Biomedicals Inc., Costa Mesa, CA,

USA) supplemented with sodium bicarbonate (2.2 g/l), glutamine (0.292 g/l) and gentamicin (4 g/l) and maintained at 28°C . Supernatants were collected weekly. After dialysing against PBS (0.01 M sodium phosphate, 0.15 M NaCl) and concentrating in an Amicon YM-10, they were finally frozen at -20°C until use.

Total IgE and specific IgE against *Anisakis simplex*

Total IgE was assessed by IMx-Method (Abbot Diagnostics, Chicago, IL, USA). Specific IgE against *A.s.* was assessed by CAP-FEIA (Pharmacia, Uppsala, Sweden). Specific IgE was considered positive with results > 0.7 kU/l.

SDS-PAGE and IgE-immunoblotting

IgE-IB was performed on serum samples from day 0 and day 30 of 20 patients with two antigens: CE and ESP. SDS-PAGE was performed with a 12% polyacrylamide separating gel and a stacking gel of 4%. A volume of 20 μl at a final protein concentration of 1 mg/ml of each extract was applied to each well. Electrophoresis was performed for 1 h at 130 V per gel for the 8 cm by 7.5 cm gels (Mini-Protein II System, Bio-Rad Laboratories, Richmond, CA, USA). Proteins were electrophoretically transferred from the separating gel to a PDVF membrane (Millipore) in a transfer buffer. After transfer from the transblot apparatus, the nitrocellulose was placed in blocking solutions for 1 h. The nitrocellulose blot was then washed four times with TTBS (TBS-Tween) and incubated with the patient's serum overnight with rocking. Patient's sera were diluted 1 : 1 v/v to 1 : 10 v/v according to specific IgE values of *A.s.* in order to handle in the IgE-IB with similar antibody concentrations. The nitrocellulose blot was again washed four times with TTBS, and alkaline phosphatase-conjugated goat anti-human IgE (1 : 2000 v/v) was added and incubated for 6 h. After an additional wash with TBS three times, the blot was developed with equal parts of 0.12% nitro-blue tetrazolium and 0.06% 5-bromo-4-chloro-3-indolyl-phosphate in 70% dimethylformamide solution in carbonate buffer (0.2 M, pH 9.8) for 15 min.

Specific IgG4, IgG, IgM and IgA

Specific antibody levels were measured by ELISA in 16 patients for day 0 and day 30 and in eight of them for 6 months. The 96 well microtitre plates (Nunc-Immuno Plate PoliSorpTM, Roskilde, Denmark) were sensitized overnight at 4°C by the addition of 1 $\mu\text{g}/\text{ml}$ of larval CE or ESP antigen diluted in a carbonate buffer to 0.1 M at pH 9.6 (100 μl per well). After washing three times with 0.05% PBS-Tween 20 (PBS-Tween), wells were blocked by the

addition of 200 µl per well of 0.1% BSA in PBS, for 1 h at 37°C. After washing, 100 µl of duplicate dilutions of sera at 1 : 100 in PBS-Tween, containing 0.1% BSA were added and incubated at 37°C for 2 h. Once the plates were washed, 100 µl per well of goat antihuman IgM, IgG, IgG4 or IgA HRP conjugate (Biosource International, Camarillo, CA, USA) at the appropriate dilution in PBS-Tween, 0.1% BSA, were incubated for 1 h at 37°C. After washing, 100 µl per well of substrate (o-phenylene-diamine; Sigma, St Louis, MO, USA) were added at 0.04% in phosphate-citrate buffer (pH 5.0) with 0.04% hydrogen peroxide. The reaction was stopped with 3 N sulphuric acid and the plates were read at 490 nm. Simultaneously, an ELISA without antigen was provided out as control. Results were expressed as ODp-ODc indices by subtracting the mean OD of the test sera once the nonspecific reaction with the BSA used in the blocking was subtracted.

Statistical analysis

Statistical analysis was performed using SPSS statistical package (SPSS for Windows 9.0, SPSS Inc., Chicago, IL, USA).

IgG, IgG4, IgA and IgM values at different times were compared with respect to their ODp-ODc values. Total IgE and specific IgE were compared after ln-transformation of the results in kU/l. Individual IB-patterns were analysed with respect to the number of bands in order to compare serum samples of day 0 and day 30 of each individual. The same analysis was performed in parallel for IB with CE and ESP of *A.s.* The mean number of bands in IgE-IB was compared for day 0 and day 30.

Statistical analysis had three objectives: (i) to compare time-kinetics of IgG, IgG4, IgM and IgA with respect to specific IgE using Wilcoxon matched-pairs signed rank test and Pearson correlation coefficients (C) after the Kolmogoroff-Smirnoff-test. (ii) To compare the number of bands in IgE-IB between day 0 and day 30 and between CE and ESP, as well as to search for an association between the number of bands, immunoglobulin levels and total IgE. For this purpose, mean number of bands were calculated for CE and ESP and the number of bands at day 30 were compared with day 0 by Wilcoxon matched-pairs signed rank test. Pearson correlation coefficients (C) were calculated between immunoglobulin levels and number of bands. (iii) To compare the mentioned parameters with respect to previous hypersensitivity to fish by using the *t*-test for equality of means, comparing patients with previous episodes of acute urticaria/angioedema with fish and those without. Correlation studies were performed using the Pearson correlation coefficient (C) to compare immunoglobulin levels and age.

RESULTS

A total of 51 serum samples were studied from 24 patients with a mean (SD) age of 46 (15.8) years.

Time kinetics of immunoglobulin isotypes and interrelationship

The time evolution of mean immunoglobulin levels is shown in Figure 1(a-f). As previously reported by our group, total IgE and specific IgE levels rise after 1 month and subsequently decrease to near basal levels (8).

Specific IgG4 against CE levels rise and decrease similar to specific IgE. A small rise in immunoglobulin levels after 1 month can also be observed for specific IgG (CE and ESP), but antibodies against CE are maintained over 6 months with no significant changes after 6 months. The behaviour of specific IgM is different with decreasing levels at day 30 and, after 6 months, with respect to day 0 values. Specific IgA antibodies do not change, with the exception of a small decrease of antibodies against ESP at 6 months with respect to day 30.

Tables 1 to 3 show the significant results of correlation studies. Total IgE and specific IgE show high correlation coefficients against each other at any time, as previously reported (9). For all other immunoglobulin classes (IgG4, IgG, IgM, and IgA), there is a positive correlation between antibodies directed against CE and those directed against ESP of the same isotype. Correlation studies between different isotypes show a positive correlation between IgG4 and IgG as well as between IgM and IgA for day 0 and day 30, if anti-CE antibodies were analysed. At 6 months, IgG4 anti-CE correlates positively with anti-CE IgA antibodies. For antibodies against ESP, only day 30 levels of IgG correlate with IgG4. A surprising finding is the high significant negative correlation between anti-ESP IgG antibodies and IgM against CE or ESP at 6 months.

IgE-Immunoblotting and relationship to immunoglobulin isotypes

Figures 2 and 3 show IgE-IB and mean number of bands. Mean number increase significantly between day 0 and day 30 for CE ($P = 0.001$) and ESP ($P = 0.006$). The most frequent bands detected in CE-IB are double bands in the area between 60 and 70 kDa ($n = 13$), double bands in the area between 35 and 40 kDa ($n = 12$) and a band between 14 and 17 kDa. The most frequent bands in ESP-IB are single bands detected at approximately 25 kDa ($n = 14$) and 14–17 kDa ($n = 6$).

There is a high correlation between the number of bands against CE and ESP ($C > 0.8$; $P < 0.01$). We also

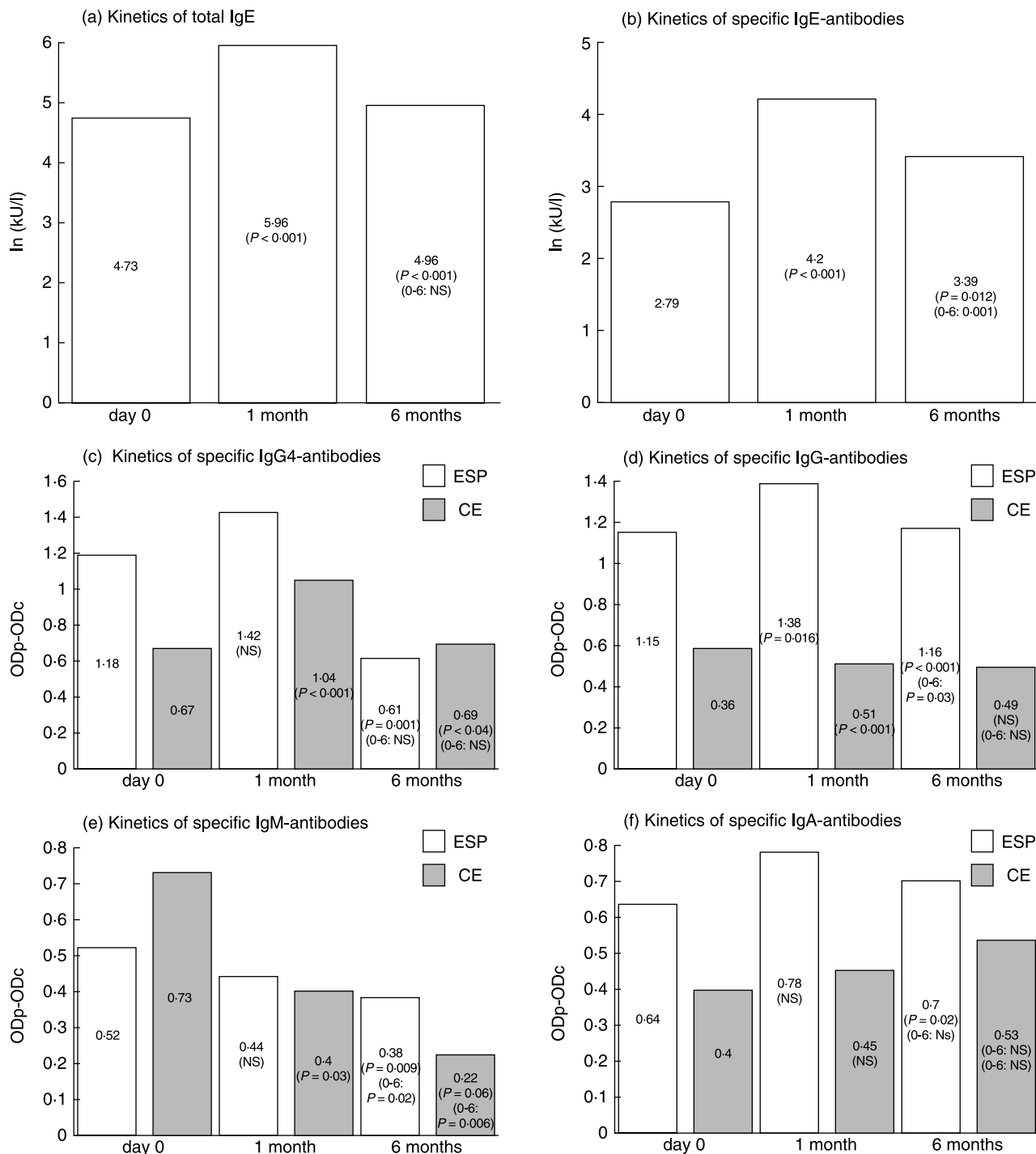


Figure 1 (a–f) Mean immunoglobulin levels of specific IgE and total IgE are expressed in kU/l after $\ln$ -transformation. Other immunoglobulin levels are expressed as ODp-ODc (see text). ESP, excretory–secretory products; CE, crude extract. Material in parenthesis (above): P for difference to previous mean levels; (below): P for difference to mean levels between day 0 and 6 months. NS, not significant.

Table 1 Correlation studies for day 0 immunoglobulin levels

	Specific	IgG4		IgG		IgM		IgA	
	IgE	CE	ESP	CE	ESP	CE	ESP	CE	ESP
Total IgE	0.88 (< 0.001)				0.48 (0.017)				
Specific IgE		0.40 (0.05)			0.58 (0.003)				
		IgG4-CE	0.48 (0.02)	0.58 (0.003)					
				IgG-CE	0.52 (0.01)				
						IgM-CE	0.8 (< 0.001)	0.68 (0.004)	
								IgA-CE	

Pearson correlation coefficients and significance (in brackets) if $P < 0.05$. Otherwise values are not shown.

Table 2 Correlation studies for day 30 immunoglobulin levels

	Specific	IgG4		IgG		IgM		IgA	
	IgE	CE	ESP	CE	ESP	CE	ESP	CE	ESP
Total IgE	0.89 (< 0.001)			0.42 (0.04)					0.58 (0.02)
Specific IgE									
		IgG4-CE	0.59 (0.002)	0.46 (0.02)					
		IgG4-ESP		0.43 (0.04)	0.55 (0.005)				
				IgG-CE	0.74 (< 0.001)				
						IgM-CE	0.78 (< 0.001)	0.52 (0.04)	
								IgA-CE	0.72 (0.002)

Pearson correlation coefficients and significance (in brackets) if $P < 0.05$. Otherwise values are not shown.

Table 3 Correlation studies for 6-month immunoglobulin levels

	Specific	IgG4		IgG		IgM		IgA	
	IgE	CE	ESP	CE	ESP	CE	ESP	CE	ESP
Total IgE	0.93 (< 0.001)								
Specific IgE								0.73 (0.04)	
		IgG4-CE	0.76 (0.01)						
				IgG-ESP		-0.82 (0.01)	-0.78 (0.02)		
						IgM-CE	0.98 (< 0.001)		
								IgA-CE	0.79 (0.02)

Pearson correlation coefficients and significance (in brackets) if $P < 0.05$. Otherwise values are not shown.

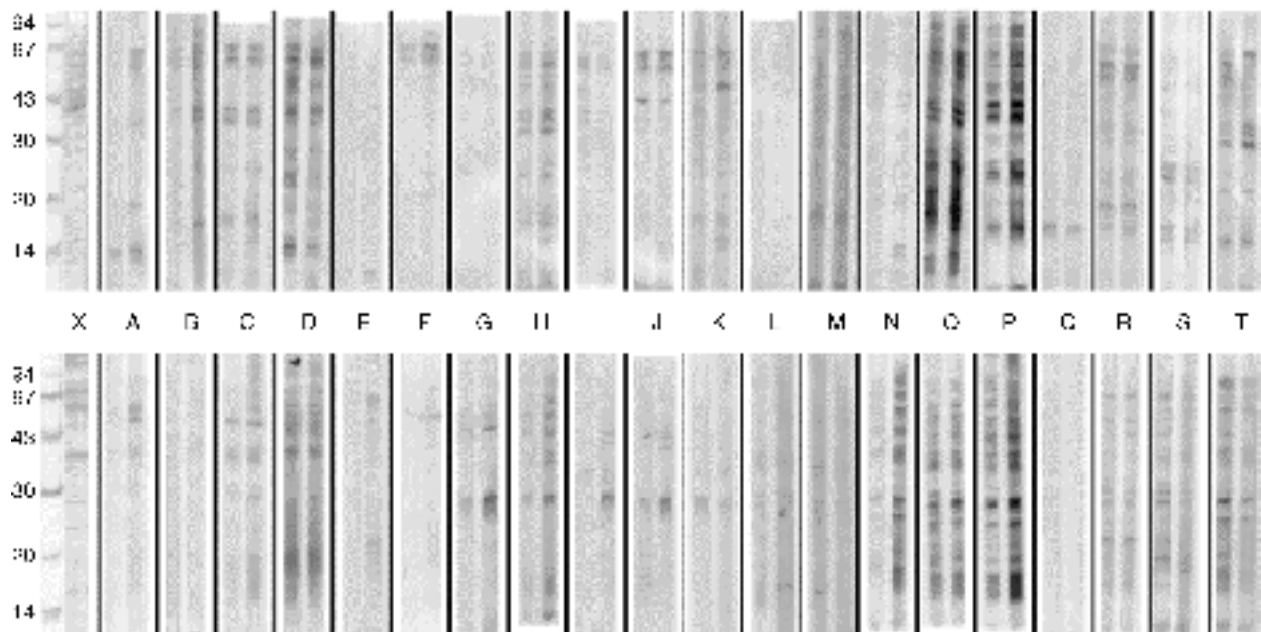


Figure 2 IgE-Immunoblotting analysis against *Anisakis simplex*. Twenty individual sera (A–T) at day 0 (left) and day 30 (right). Analysis with crude extract (above) and excretory–secretory products (below) showed not only a stronger appearance at day 30, but possibly also an increase in the antigens recognized against *Anisakis simplex* by a given individual with time.

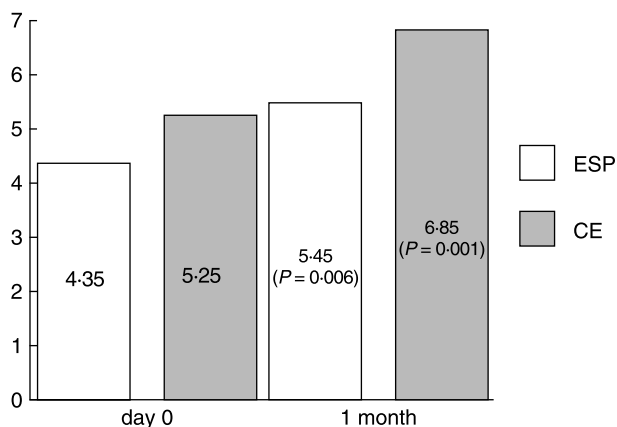


Figure 3 Number of bands in IgE immunoblotting. ESP, antibodies against excretory-secretory products; CE, Antibodies against crude extract of *A.s.* P for difference to previous mean level.

found a positive correlation between the number of bands and total, as well as specific, IgE (C between 0.47 and 0.67; $P < 0.05$). For other immunoglobulin classes, there is only a moderate positive correlation against day 0 anti-CE IgM and against day 30 anti-ESP IgG4 antibodies.

Immunoglobulin levels, age and previous fish-associated hypersensitivity reactions

Eight of 24 patients reported one or more previous acute allergic hypersensitivity symptoms related to fish. We could not detect any significant differences in immunoglobulin levels or number of bands between patients with or without previous allergic symptoms related to fish.

A significant correlation with regard to age could only be stated against anti-CE IgM antibodies at day 0 ($C = -0.52$; $P = 0.037$). In parallel, this negative correlation is similar against anti-ESP IgM antibodies at day 0 ($C = -0.47$; $P = 0.065$). A nearly significant positive correlation could be detected against anti-CE IgG antibodies at 6 months ($C = 0.6$; $P = 0.068$).

DISCUSSION

Gastric anisakiasis is a well-known entity, being common in Japan, where up to 1000 cases are diagnosed every year (10,11). Eating habits are the main cause, as ingestion of raw or undercooked fish is a risk-factor for contact with live larvae of *A.s.* However, several publications from countries all over the world describe clinical observations with the same diagnosis (12–17). A wide range of publications deal with immunologic features in different animal models (18–21).

Human gastro-allergic anisakiasis is an acute parasitism in which type I hypersensitivity symptoms are coincident with the time of the nematode contact with the gastric mucosa. Thus, the allergic symptoms are an indicator of an immediate onset of the immunological reaction against the parasite. Prior to any interpretation of results, we have to focus on the following peculiarities of this entity: First, we deal with an acute parasitism, which is mainly self-limiting in a few hours or days (6). Second, patients are all supposed to have had at least one previous contact with the clinical allergen of *Anisakis simplex*, but many contacts with other *A.s.* antigens because of the fish-eating habits and prevalence of fish parasitism in our area (22–24). In this natural experiment, we wanted to search for peculiarities of early immunoglobulin isotype response and their kinetics.

It is well known that helminth infections lead to a dramatic induction of the Th2 lymphocyte subset, resulting in stimulation of IgG4 and IgE isotypes (25). In recent years, immunohelminthological investigations have focused mainly on this Th2 response, but we should not neglect coexisting Th1 lymphocyte stimulation, as has been demonstrated in human helminth infection, such as human hydatid disease or in experimental *A.s.* infections in laboratory animals (18,19,26–29).

Allergic manifestations are seldom observed in helminthosis and blocking antibodies or the ratio of IgE to IgG4 have been proposed in determining the expression of allergic symptoms (30,31). The peculiar acute parasitism by *A.s.* accompanied by allergic symptoms reflects a small subgroup of otherwise mainly undiagnosed gastric anisakiasis without allergic manifestations (16,32). It has been stated that mast cell degranulation has never been shown to be beneficial to the allergic individual (33). This is in contrast to our idea that the systemic allergic reaction reflects the local immunologic reaction against the penetrating nematode, thus expelling it with more efficacy, rendering gastro-allergic anisakiasis a mainly self-limited infestation of some hours duration (5,6).

We have shown that anti-*Anisakis* antibodies in our patients belong to all isotypes studied, reflecting a broad immunologic stimulation involving a Th2 as well as Th1 lymphocyte response. Our selected patients displayed an IgE-mediated hypersensitivity reaction supposed to reflect a previous sensitization. Thus, the detected immune features belong at least to a secondary Th2 memory response. There are however, some aspects of the kinetics of specific antibodies that are interesting to underline: despite a short, temporary contact with antigens or allergens, IgE and IgG4 as well as IgG and IgA antibodies show the highest values after 1 month. Only specific IgM levels differ in this respect, showing the highest levels at day 0, and falling continuously over the next 6 months. This would not be surprising as in

every immunological response, it is the first isotype to be produced and detected. However, two aspects are worth consideration. First, it is the rapid onset of IgM production within 24 h (median time of the first serum sample is 10.5 h; data not shown). Second, if the allergic reaction represents the onset of a secondary reaction mediated by IgE antibodies, the immunological response cannot be considered as a simple memory reaction. Simultaneously, a new primary immunological response is underway including not only a boost response to cross-reacting antigens with which the patient was in contact before, during or after the current gastro-allergic episode, but also against new antigens or epitopes of *A.s.* that have not been the target of previous immune reactions. This underlines our previous observation that the allergic reaction in the course of the contact with the live parasite has to be interpreted as an IgE memory response paralleling the parasite's new primary stimulation of the host immune system (8,9). One way of interpreting the data would place all the immune features as a primary response with a rapid detection of IgM antibodies, but also a rapid production of IgE antibodies able to initiate an allergic reaction and detectable specific antibodies in only a few hours.

Kinetics of specific as well as total IgE show an important rise of both parameters after 1 month, falling afterwards to near basal levels. This reflects a polyclonal expansion that accompanies the production of specific IgE antibodies against new antigens of *A.s.* as demonstrated by means of IB. Thus, the live parasite induces a mechanism by which new IgE antibodies are produced, as a consequence of the penetration of the gastric mucosa with the help of secreted proteases (34,35). Surface body proteins of *A.s.* located near the secreted immunogenic proteases are thus bystander or 'third-party' antigens (25). This micro milieu is also the origin of the production of other immunoglobulin isotypes. It is interesting that the only antibodies that do not change significantly over the time studied are those of the IgA isotype directed against CE. We would like to speculate that this reflects a basal luminal immune response against 'dead' *A.s.* antigens as a regular food antigen in frequently parasitized fish.

Antigens from *Anisakis simplex*, as from other ascarids, have a great potential for cross-reactivity: circulating antibodies against *A.s.* do not just include antibodies produced in response to the current parasite infection. *A.s.* antigens may boost responses to other cross-reacting antigens as from other ascarids (21,36). However, *Ascaris* or *Toxocara* infection is rare in our area, and the low prevalence of Echinococcosis as the only significant parasite infection in our area would explain such a phenomenon in only particular cases. On the other hand, cross-reactivity of *A.s.* has been reported with food antigens, such as crustaceans,

and inhalant allergens, such as house dust mites and cockroaches (37,38).

In IgE-IB we could compare the number of detected bands. There were more antibodies directed against CE than against ESP, which is in accordance with both the mass of antigenic components and probably also with the number of possible antigenic proteins. On the other hand, a study with sera of patients after gastric anisakiasis revealed that sera from the early phase of the infection hardly recognized the larval somatic extract antigen, ESP products being one of the first targets of the immune response (27). This may be explained by the fact that these patients had not been exposed to previous acute parasitism by this nematode, in contrast to our sensitized patients, whose gastro-allergic episode reflects a previous primary response against the live *A.s.*

The results of our correlation studies demonstrate a parallel immunologic stimulation of antibodies against CE and ESP in each Ig-isotype. In a previous report with *A.s.* allergic patients, no correlation between IgE levels and IgG, IgM and IgA levels was observed (3). We found a positive correlation of anti-ESP antibodies of the IgE and IgG4 isotype after 1 month, the time of highest specific immunoglobulin levels. This reflects the known parallel production of IgE and IgG4 that compete for the same antigenic targets (30,31,39).

In conclusion, our data demonstrate that the allergic reaction to the early acute parasitism by *Anisakis simplex* is a consequence of an immediate IgE memory response with a simultaneous new primary response with participation of Th1 and Th2 lymphocyte subsets. The allergic reaction itself should produce a local Th2 milieu, as it has been shown that mast-cell degranulation is a source of IL-4 (40). Nevertheless, the evidence suggests that the parasite's stimulatory molecules give rise to a immune response involving both Th subsets.

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