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The *Anisakis simplex* Ani s 7 major allergen as an indicator of true *Anisakis* infections

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Summary

Ani s 7 is currently the most important excretory/secretory (ES) *Anisakis simplex* allergen, as it is the only one recognized by 100% of infected patients. The allergenicity of this molecule is due mainly to the presence of a novel $CX_{17–25}CX_{9–22}CX_8CX_6$ tandem repeat motif not seen in any previously reported protein. In this study we used this allergen as a model to investigate how ES allergens are recognized during *Anisakis* infections, and the usefulness of a recombinant fragment of Ani s 7 allergen (t-Ani s 7) as a marker of true *Anisakis* infections. The possible antigenic relationship between native Ani s 7 (nAni s 7) from *Anisakis* and *Pseudoterranova decipens* antigens was also investigated. Our results demonstrate that nAni s 7 is secreted and recognized by the immune system of rats only when the larvae are alive (i.e. during the acute phase of infection), and that this molecule is not present in, or is antigenically different from, *Pseudoterranova* allergens. The t-Ani s 7 polypeptide is a useful target for differentiating immunoglobulin E antibodies induced by true *Anisakis* infections from those induced by other antigens that may cross-react with *Anisakis* allergens, including *P. decipiens*. The results also support the hypothesis that the Ani s 7 major allergen does not participate in maintaining the antigenic stimulus during chronic infections.

Keywords: allergen, Ani s 7, *Anisakis*, IgE, *Pseudoterranova*

Introduction

Anisakiasis (= Anisakiosis) is an important disease produced by ingestion of raw or undercooked fish infected with third-stage (L3) *Anisakis* spp. larvae [1]. Since the first case reported by van Thiel *et al.* in 1960 [2], thousands of cases of human anisakiosis have been reported worldwide, although infections occur mainly in Japan and some European countries [3–5]. Infection by *Anisakis* induces stimulation of both T helper type 1 (Th1) and Th2 responses, and provokes a strong specific immune response by all antibody isotypes [immunoglobulin (Ig)E, IgG, IgA and IgM] [5]. The most frequent symptom reported in acute infections is abdominal pain of variable location and intensity, which follows a variable incubation period of 4–48 h after infection by the parasite [6]. However, more than 10% of gastrointestinal anisakiasis may be accompanied by allergic symptoms [7–9], ranging from intermediate urticaria to severe anaphylaxis [5]. In addition, several studies have detected the presence of anti-*Anisakis* IgE antibodies in more than 10% of healthy subjects [10], suggesting the existence of a large number of infected patients who do not develop clinical symptoms.

Unlike in marine mammals, *Anisakis* larvae do not usually reach the adult stage in humans and the larvae die during the course of 3 weeks after infection [11]. Consequently, it is expected that the immune response against *Anisakis* allergens from third- and/or fourth-stage larvae (L3/L4) occurs in response to two consecutive antigenic stimuli: (i) the excretory/secretory (ES) and cuticle antigens while the larvae is alive; and (ii) the cuticle and protease-resistant somatic and ES antigens, after the larvae die. Previous studies have shown that the *Anisakis* ES allergens are the most clinically important, as they are targeted by most of the anti-*Anisakis* IgE antibodies induced during infections by this parasite [12]. However, there are no data available to indicate whether these antigens stimulate the immune system only during the short period of time after infection in which the larvae remain alive, or also after the death of the larvae. Such data may be important in order to establish which *Anisakis* allergens cause the pathological changes observed during the acute and chronic stages of the infection.

Another characteristic of *Anisakis*-induced allergy is that either the ES allergens [12] or the allergens stored inside dead larvae [13–15] are not able to induce clinical symptoms

when administered to previously sensitized patients by the oral route. These results suggest that: (i) immunization against clinically relevant *Anisakis* allergens is only possible during the course of an active infection; and (ii) that these allergens are destroyed during the digestion process. Accordingly, it would be expected that one or more *Anisakis* ES-specific allergens could be used as a marker of *Anisakis* infections, which is of clinical relevance to allow the differentiation of IgE antibodies induced by the parasite (true infection) from others induced by cross-reacting allergens frequently present in nature (false infection).

Several ES and somatic *Anisakis* allergens have been cloned and characterized in recent years (Allergen Nomenclature Sub-Committee; http://www.allergen.org). Among these, Ani s 1 (21 kDa) and Ani s 7 (139 kDa) are probably the most important major ES allergens described, as they were reported to be recognized by 85% and 100% of infected patients respectively [16,17]. Ani s 2 (paramyosin; 100 kDa) and Ani s 3 (tropomyosin; 41 kDa) are somatic *A. simplex* allergens that cross-react with other common allergens [18,19]. Finally, other cloned *A. simplex* allergens such as Ani s 4 (cystatin, 9 kDa), Ani s 6 (serine protease inhibitor, 7 kDa) and three SXP/RAL-2 family proteins, named Ani s 5 (15 kDa), Ani s 8 (15 kDa) and Ani s 9 (14 kDa), are all minor ES allergens recognized by fewer than 50% of infected patients [20–23].

Given the high potential of the Ani s 7 ES allergen as a target for *Anisakis*-induced allergy, and for immunodiagnosis of human anisakiasis [17], we used this allergen as a model to investigate how ES allergens are recognized during the *Anisakis* infections, and to evaluate the usefulness of a recombinant fragment of the Ani s 7 allergen (t-Ani s 7) as an indicator of true *Anisakis* infections.

Material and methods

Animals

Male Wistar rats weighing approximately 250 g at the start of experiments and Balb/c mice (4–6 months old) were obtained from the animal facilities of the University of Santiago de Compostela. All animals were provided with water and food *ad libitum*, and adequate measures were taken to minimize pain or discomfort in experimental animals. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC and RD 1201/2005) on the protection of experimental animals, and approved by the Ethics Committee of the University of Santiago de Compostela.

Obtainment of the parasites and parasite crude extracts

Third-stage larvae (L3) of *A. simplex* were extracted manually from the viscera and body cavity of blue whiting (*Micromesistius poutassou*) purchased from a local market. *Pseudoterranova decipiens* L3 were extracted manually from fillets of naturally infected monkfish (*Lophius piscatorius*) and washed thoroughly in water. The larvae were then washed with phosphate-buffered saline (PBS; pH 7·2) and their integrity was checked under a stereomicroscope. Crude soluble extract (CE) from third-stage larvae (L3) of *P. decipiens* and *A. simplex* were prepared as described previously by Perteguer and Cuéllar [24] and Lorenzo *et al.* [25] respectively. The native Ani s 7 (nAni s 7) allergen was captured in enzyme-linked immunosorbent assay (ELISA) plates (see below) with monoclonal antibody (mAb) UA3.

Monoclonal antibodies

The mAb UA3 (IgG1/k) recognizing the Ani s 7 allergen [17,26] was purified from UA3 cell culture supernatants by affinity chromatography on protein-G Sepharose (Sigma-Aldrich, Madrid, Spain), according to the supplier's instructions.

Obtainment of *Anisakis simplex* Ani s 7 major allergen

An *Anisakis* cDNA library was screened with mAb UA3, and a cDNA clone (rAni s 7) encoding a 1096-amino acid fragment of Ani s 7 was identified. The encoded sequence (rAni s 7) has no known sequence homology, but as a distinctive characteristic it comprised 19 repeats of a novel $CX_{17–25}CX_{9–22}CX_8CX_6$ tandem repeat motif not seen in any previously reported protein sequence. An internal ^{435}Met-^{713}Arg fragment of the rAni s 7 (t-Ani s 7) was expressed in *Escherichia coli* [17] and used as target in an indirect ELISA (see below).

Sera from experimental infections and immunizations

The anti-*Anisakis* sera for kinetic studies (primary infection) were obtained from 16 Wistar rats inoculated intraperitoneally with either five live L3 larvae (eight rats; group 1) or five dead L3 larvae, killed by freezing at −30°C for 12 h (eight rats; group 2). Blood samples of the anaesthetized rats were collected by careful bleeding from the ophthalmic plexus vein with a capillary tube on day 5 before inoculation, and on days 8, 15, 22, 29, 36, 43, 63, 70, 75 and 90 post-infection (p.i.). On day 115 p.i., the Wistar rats from group 2 were reinoculated intraperitoneally with five L3 *A. simplex* dead larvae (four rats) or with five live larvae (four rats), and the animals were bled on days 3, 7, 37 and 65 post-reinfection, when all animals were exsanguinated by cardiac puncture. All the sera from the experimental animals were stored at −80°C until assayed.

For cross-reactivity experiments with *P. decipiens*, BALB/c mice were immunized by intramuscular injection of a single dose of 1 ml of *A. simplex* or *P. decipiens* CE antigens in Freund's complete adjuvant (1 mg/ml in final volume) in the

quadriceps muscle at 0·5 cm above the knee. The mice were bled under general anaesthesia from the retro-orbital plexus 30 days after immunization and the sera obtained were stored at −80°C until assayed.

The ELISA methods to determine kinetics of anti-*Anisakis* antibodies

Coupling of ELISA plates with antigens and antibodies. Parasite-specific antibody isotypes were determined by indirect ELISA and by UA3-capture ELISA (UA3-ELISA) methods. For indirect ELISAs, 96-well microtitre plates were coupled overnight at 4°C with 100 µl of PBS containing 5 µg/ml of *Anisakis* CE antigen or 100 µl of 0·1 M Tris buffer pH 10·5 containing 0·6 µg/ml of recombinant t-Ani s 7 antigen, washed three times with PBS, and then blocked for 1 h at 37°C with 200 µl of Tris-buffered saline containing 0·2% Tween 20 (TBS-T) and 1% dry skimmed milk (TBS-T-SM), or with 200 µl of TBS-T containing 1% bovine serum albumin (BSA) (TBS-T-BSA) when biotinylated antibodies were used.

For the UA3-capture ELISA, the plates were coated overnight with UA3 purified Ig (1·4 µg/well) in PBS, and washed and blocked as above. The plates were then aspirated and 50 µg of *A. simplex* CE in 100 µl of TBS-T-SM or TBS-T-BSA was added to each well and incubated for 1 h at 37°C.

Specific incubations. For determination of IgE and IgA isotypes, previously coupled ELISA plates were incubated for 2 h at 37°C with experimental rat sera (tested in duplicate) diluted 1:4 in TBS-T-SM (IgE) or 1:100 in TBS-T-SM (IgA). The plates were washed with TBS-T, then incubated for 1 h at 37°C with fluorescein isothiocyanate (FITC)-labelled mouse anti-rat IgE (AbD Serotec, Oxford, UK; 100 µl/well, dilution 1:500) or FITC-labelled mouse anti-rat IgA (Serotec Ltd, Oxford, UK; 100 µl/well, dilution 1:100). After further washing with TBS-T, the captured Ig-FITC was detected by incubation for 1 h at 37°C with peroxidase-labelled rabbit anti-FITC polyclonal antibodies (Abcam, Cambridge, UK; 100 µl/well, dilution 1:5000). Finally, the plates were washed again with TBS-T; buffered H_2O_2 and *o*-phenylenediamine (SigmaFast OPD; Sigma–Aldrich) were used as the substrate for peroxidase. The optical density (OD) was measured at 492 nm. A negative control serum (pooled sera obtained 5 days before inoculation with the *Anisakis* larvae) was included in each assay and the OD value subtracted from the corresponding values obtained for test sera.

For determination of IgM or IgG isotypes (IgG1, IgG2a, IgG2b or IgG2c), experimental rat sera diluted 1:100 in TBS-T-BSA (100 µl/well; tested in duplicate) were added to previously coupled ELISA plates and incubated for 2 h at 37°C. The plates were then washed with TBS-T and incubated for 1 h at 37°C with an appropriate dilution of the suitable biotinylated mouse anti-rat mAb in TBS-T-BSA (BD Bioscience Pharmingen, Madrid, Spain). The dilutions used were 1:100 for anti-rat IgM, 1:200 for anti-rat IgG2b, 1:400 for anti-rat IgG2a, 1:500 for anti-rat IgG2c and 1:1000 for anti-rat IgG1. At the end of the incubation period the plates were washed with TBS-T; 100 µl/well of Neutravidin™-horseradish peroxidase (Pierce Biotechnology, Rockford, USA) was applied at a dilution of 1:10 000, and the plates incubated again for 1 h at 37°C. Finally, the plates were washed with TBS-T and the reaction was revealed with buffered H_2O_2 and *o*-phenylenediamine, as described above for IgE and IgA isotypes. As above, the OD value obtained for the preimmune serum was subtracted from the corresponding values obtained for test sera.

Cut-off calculations. The cut-off value for each Ig isotype was calculated as four times the standard deviation (+4) of the mean OD value obtained after individual testing of all rat sera collected 5 days before inoculation with the *Anisakis* larvae.

Indirect ELISA for the study of t-Ani s 7 genus specificity

The 96-well microtitre plates were reacted overnight at 4°C with 100 µl of PBS containing 5 µg/ml of *Anisakis* or *Pseudoterranova* CE antigens, or 0·6 µg/ml of recombinant t-Ani s 7 antigen in 0·1 M Tris buffer pH 10·5. Plates were then washed with TBS-T and non-specific binding sites were blocked by incubation (1 h at 37°C) with TBS-T-SM. After further washing, 100 µl of a 1:100 dilution of mouse immune sera (tested in quadruplicate) was added and the plates were incubated for 2 h at 37°C. After another washing step, specific IgG1 antibodies were detected with 100 µl/well of peroxidase-labelled goat anti-mouse IgG1 polyclonal antibodies (Caltag Laboratories, San Francisco, CA, USA; dilution 1:1000). The reaction was developed with buffered H_2O_2 and *o*-phenylenediamine and read at 492 nm, as described above. Sera from non-immunized BALB/c mice were used as negative controls.

Statistical analyses

Results from different groups were compared by a paired *t*-test. Differences were considered significant at $P < 0{\cdot}05$. Data analysis was performed with the GraphPad InStat program (GraphPad Software Inc., San Diego, CA, USA).

Results

In vivo recognition of ES *Anisakis* allergens

To investigate how the *Anisakis* ES antigens are recognized in natural infections, Wistar rats were inoculated intraperitoneally with dead or live *Anisakis* L3 larvae and the kinetics of specific antibodies produced against three target antigens:

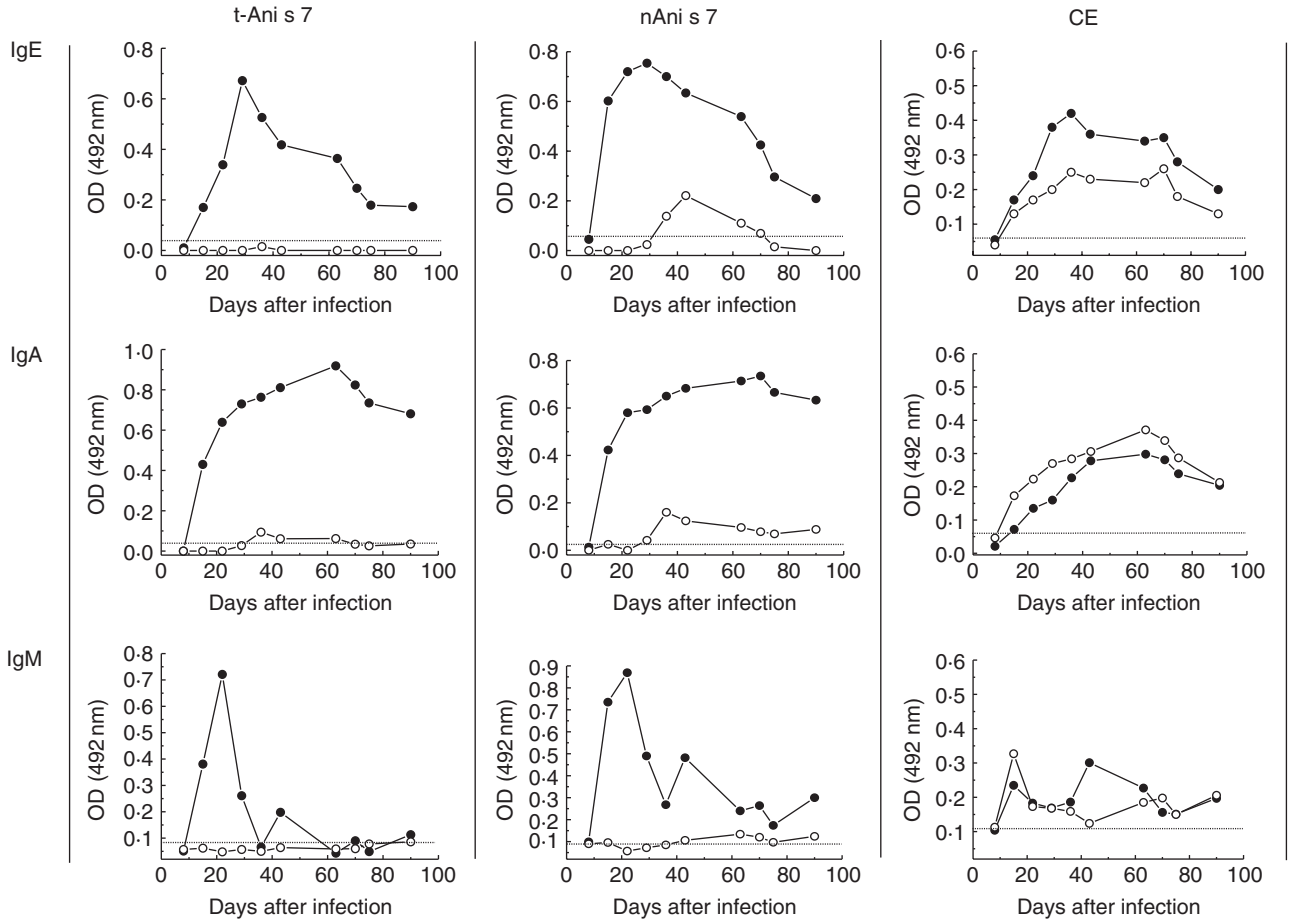

Fig. 1. Kinetics of immunoglobulin (Ig)E, IgA and IgM antibodies induced in rats after intraperitoneal inoculation with live (closed circles) or dead (open circles) L3 *Anisakis* larvae. Enzyme-linked immunosorbent assays were carried out with recombinant Ani s 7 allergen (t-Ani s 7), native Ani s 7 (nAni s 7) or crude extract (CE) as target antigens. Each serum sample was tested in duplicate. Cut-off values are represented by a horizontal dashed line.

t-Ani s 7 (internal ^{435}Met-^{713}Arg recombinant fragment of the nAni s 7) [17], nAni s 7 and *Anisakis* CE were tested. The antibody levels were measured by indirect ELISA with immobilized t-Ani s 7, by capture ELISA with immobilized mAb UA3 capturing nAni s 7 from *Anisakis* CE and by an indirect ELISA containing immobilized *Anisakis* CE. As shown in Fig. 1, the t-Ani s 7 antigen was recognized only by IgE antibodies induced by live larvae, and the IgE response peaked on day 30 p.i. When nAni s 7 was used as target, the IgE response induced by live larvae also peaked on day 30 p.i., but compared with t-Ani s 7 the IgE response induced by dead larvae was low and peaked on day 42 post-immunization. As far as the IgE response against *Anisakis* CE is concerned, a discrete response was induced by either live or dead larvae covering the entire observation period from the second week of infection/immunization. The pattern of the anti-*Anisakis* IgA antibody response was very similar to that obtained for IgE, except that: (i) the maximum response peaked later (at about 2 months after infection); (ii) there was very little IgA response with t-Ani s 7 from the animals immunized with dead larvae; and (iii) the anti-t-Ani s 7 IgA antibodies decreased more slowly than IgE antibodies (Fig. 1). Considering IgM antibodies, good responses against t-Ani s 7 and nAni s 7 were observed only in sera from infected animals. This response peaked by day 21 after infection and then decreased quickly. Finally, the levels of anti-CE IgM antibodies were low throughout the entire observation period with rats inoculated either with live or dead L3 *Anisakis* larvae (Fig. 1).

The data in Fig. 2 show the kinetics of IgG antibody responses. Considering the four IgG rat isotypes and the three antigens assayed, we observed three main characteristics of these antibody responses: (i) high IgG1, IgG2a and IgG2b antibody responses against t-Ani s 7 antigen in infected rats, with minimal or no IgG antibodies induced against this antigen in animals immunized with dead larvae; (ii) high IgG1 levels against nAni s 7 antigen induced with either live or dead larvae; and (iii) low IgG2c responses induced by either live or dead larvae against t-Ani s 7 and nAni s 7, which contrasted with the relatively good biphasic

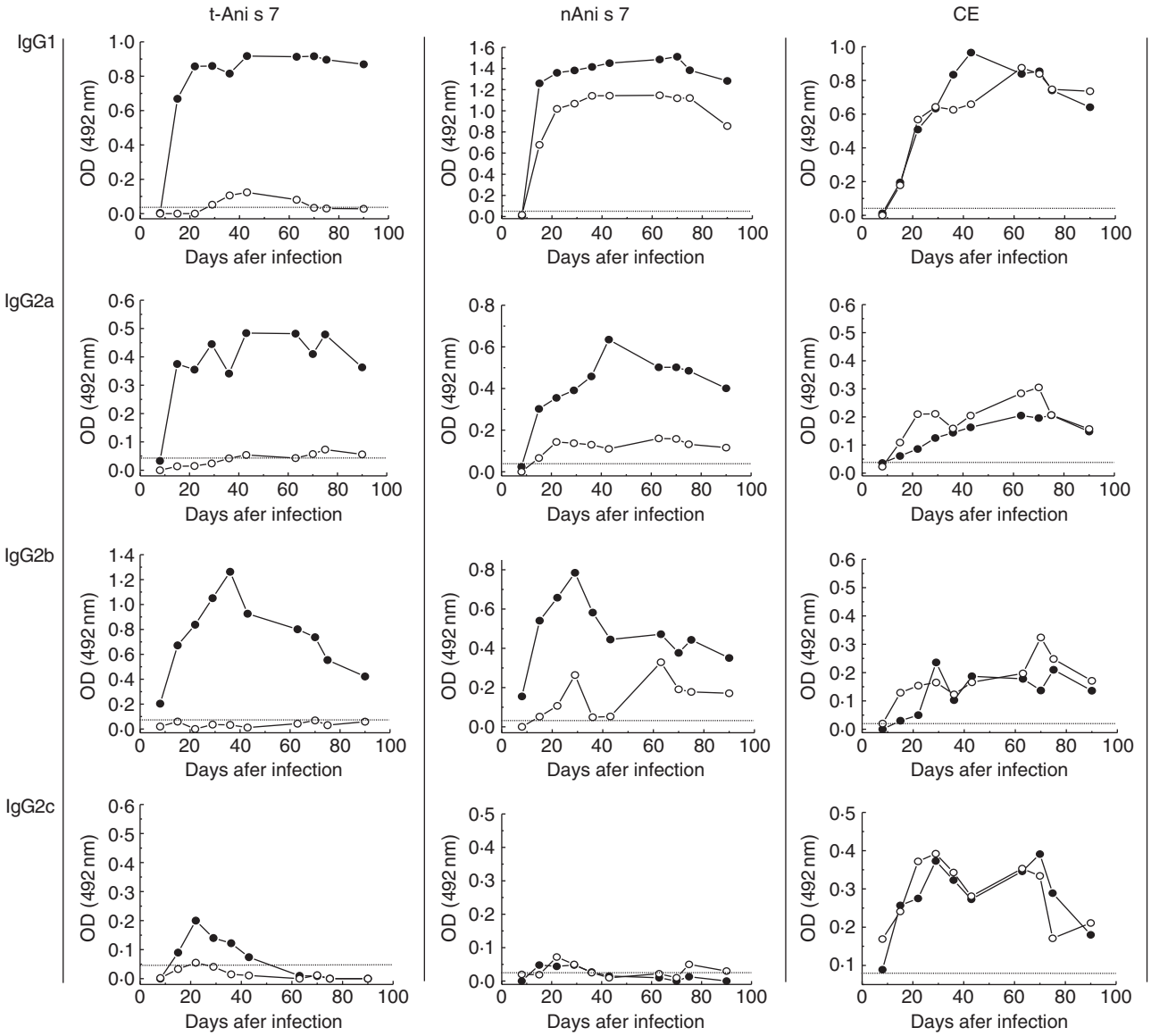

Fig. 2. Kinetics of immunoglobulin (Ig)G (IgG1, IgG2a, IgG2b and IgG2c) antibodies induced in rats after intraperitoneal inoculation with live (closed circles) or dead (open circles) L3 *Anisakis* larvae. Enzyme-linked immunosorbent assays were obtained with recombinant Ani s 7 allergen (t-Ani s 7), native Ani s 7 (nAni s 7) or crude extract (CE) as target antigens. Each serum sample was tested in duplicate. Cut-off values are represented by a horizontal dashed line.

responses obtained against the antigens contained in the *Anisakis* CE. In addition, after reaching maximal values, anti-t-Ani s 7 and anti-nAni s 7 IgG1 antibodies remained stable throughout the entire observation period (Fig. 2: t-Ani s 7 and nAni s 7).

The IgE and IgG1 secondary antibody response induced against t-Ani s 7 in rats inoculated previously with dead larvae and then reinoculated with either dead or live larvae was also investigated (Fig. 3). A similar pattern to that obtained in the primary response was observed, with good levels of IgE and IgG1 induced by live L3 *Anisakis* larvae and poor responses induced in rats reinoculated with dead larvae.

Testing of the genus specificity of the t-Ani s 7 polypeptide

The above results, showing that the antibodies induced by the antigens contained in dead *Anisakis* larvae did not recognize the t-Ani s 7 polypeptide, suggest strongly that: (i) the antigenic stimulation by ES antigens ceases after the infecting larvae die; and (ii) the t-Ani s 7 polypeptide does not have any epitopes in common with other *Anisakis* antigens, and thus may be a candidate target for use as a specific marker of *Anisakis* infections. However, a doubt arises as to whether this polypeptide is sufficiently specific to enable

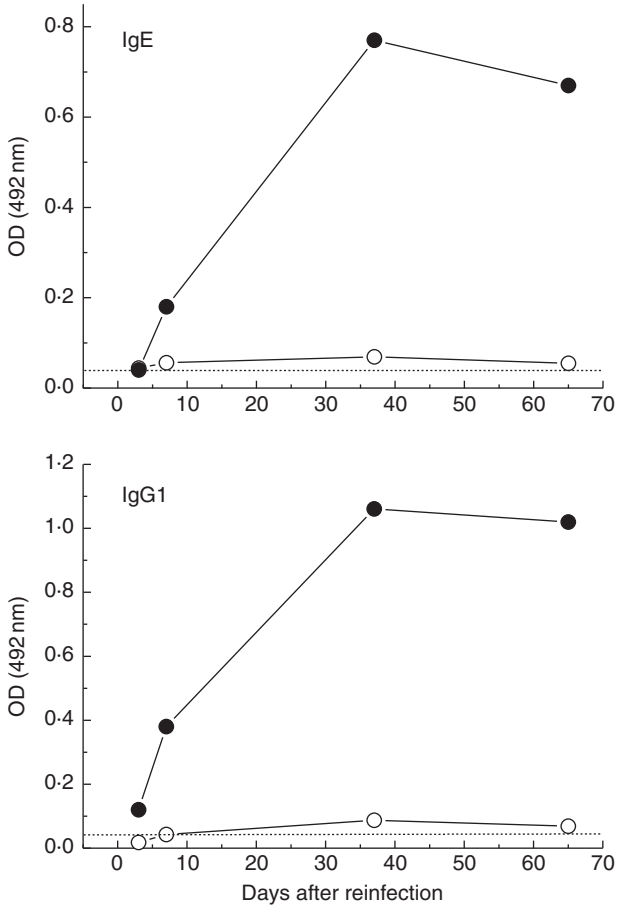

Fig. 3. Kinetics of immunoglobulin (Ig)G1, and IgE antibodies induced in rats after intraperitoneal challenge (secondary response) with live (closed circles) or dead (open circles) L3 *Anisakis* larvae. All the enzyme-linked immunosorbent assays were carried out with recombinant Ani s 7 allergen (t-Ani s 7) as target antigen. Each serum sample was tested in duplicate. Cut-off values are represented by a horizontal dashed line.

differentiation between infections provoked by *Anisakis* or by other members of the Anisakidae family, notably *P. decipiens*. To address this question, recognition of the t-Ani s 7 polypeptide by serum IgG1 antibodies from mice immunized with CEs from *A. simplex* and *P. decipiens* was compared by indirect ELISA. The CE antigens from *A. simplex* and *P. decipiens* were also used as positive controls for cross-reactivity in ELISA. The results showed that the antibody response induced by *Pseudoterranova* and *Anisakis* CEs were cross-reactive, whereas only IgG1 antibodies from mice immunized with *A. simplex* CE were recognized in indirect ELISA with the t-Ani s 7 antigen as target ($P < 0{\cdot}0001$) (Fig. 4).

Discussion

In this study we have shown that rats inoculated intraperitoneally with dead *Anisakis* larvae failed to produce significant amounts of IgE or other antibody isotypes against the highly specific *Anisakis* t-Ani s 7 recombinant polypeptide, comprising the internal ^{435}Met-^{713}Arg of the nAni s 7 major allergen [17]. Indeed, these rats were also unable to produce significant amounts of anti-t-Ani s 7 antibodies after reinoculation with more dead L3 *Anisakis* larvae. Together these findings suggest strongly that: (i) the antigenicity of the nAni s 7 molecule, or at least that corresponding to the t-Ani s 7 fragment, is destroyed inside the dead larvae; and (ii) after the larvae die, there are no remaining *Anisakis* antigens that share the same epitopes with the t-Ani s 7 polypeptide. These results are consistent with a scenario in which Ani s 7, and probably other relevant allergens produced and accumulated in the excretory cell of the parasite while it remains alive, are cleaved inside the L3 dead larvae by endogenous proteases until the outer cuticle becomes damaged. Accordingly, we hypothesize that nAni s 7, and probably other *Anisakis* ES allergens, stimulate the immune system only during the short time during which the infecting larvae remain alive (i.e. during the acute phase of infection), whereas other *Anisakis* antigens (e.g. protease-resistant somatic and cuticle antigens) probably maintain the antigenic stimulus during the chronic stage of anisakiasis. This hypothesis is consistent with previous findings that have shown that reactivity against *Anisakis* larvae is maintained during the chronic phase of anisakiasis when the larvae have already been destroyed and only cuticle debris remains [27].

The present finding that no antigen present in dead *Anisakis* larvae was capable of inducing antibodies against the t-Ani s 7 recombinant sequence is of clinical relevance, as

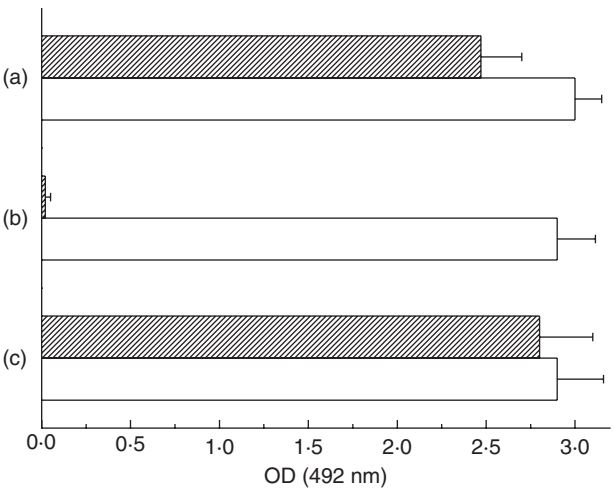

Fig. 4. Analysis of cross-reactivity between crude extract (CE) antigens of *Anisakis simplex* and *Pseudoterranova decipiens*. Mice were injected intramuscularly with CE antigens from *P. decipiens* (crossed bars) or *A. simplex* (white bars) and their IgG1 serum antibodies were tested (in duplicate) by enzyme-linked immunosorbent assay against *P. decipiens* CE antigen (a), recombinant Ani s 7 allergen (t-Ani s 7) (b) and *A. simplex* CE antigen (c). Values are represented as mean optical density plus standard deviation.

it confirms that all epitopes contained in this sequence are specific to the nAni s 7 ES allergen. This suggests the possibility of using this polypeptide to differentiate patients with true acute or chronic *Anisakis* infections (i.e. with a positive IgE response against t-Ani s 7) from those with false anti-*Anisakis* IgE antibodies induced by other antigens that may cross-react with some *Anisakis* allergens. Furthermore, as the anti-*Anisakis* antibody response against the t-Ani s 7 polypeptide was genus-specific (see Fig. 4), differentiation between *Anisakis* and *Pseudoterranova* infection may also be possible. Although human *Anisakis* infections are much more prevalent, pseudoterranovosis is currently arising as an emerging infection in some countries where consumption of raw fish in different dishes such as sushi, sashimi and ceviche is becoming popular [28,29].

In contrast to the failure of the *Anisakis* dead larvae to induce a good antibody response against the polypeptide t-Ani s 7 in rats, dead larvae were able to induce a strong IgG1 response and slight IgE, IgA, IgG2b and IgG2c responses when nAni s 7 was used as target antigen in capture ELISA. These antibodies were probably produced by other *Anisakis* protease-resistant antigens (e.g. somatic and/or cuticle antigens) with some common epitopes, e.g. *O*-glycans, which we have demonstrated previously to be involved in the formation of cross-reacting anti-nAni s 7 antibodies [30]. Alternatively, this antibody response may be due to the presence of one or more protease-resistent epitopes in the nAni s 7 allergen, located in a different part of that corresponding to the t-Ani s 7. Using CE *Anisakis* antigens as target (Fig. 1: CE and Fig. 2: CE), we observed that dead *Anisakis* larvae were able to induce a very similar antibody response to that induced by the live larvae, which indicates that, unlike Ani s 7, some *Anisakis* antigens are resistant to enzymatic cleavage inside the larvae, and are consequently able to stimulate the host immune system after the larvae are destroyed.

Previous studies on *Anisakis*-induced allergy have suggested that the adverse reactions observed in some patients after ingestion of well-cooked or even canned fish could be related to *Anisakis* allergens that are resistant to heat and/or pepsin treatments [22,31,32]. However, these studies did not establish whether the IgE antibodies reacting with heat/enzyme resistant *Anisakis* allergens were induced by direct contact with these allergens at a gastrointestinal level (i.e. without previous infection), or whether they were produced as a consequence of previous infection by the parasite. The use of the t-Ani s 7 polypeptide to detect true *Anisakis* infections may help allergologists to solve this question, which is important in order to establish preventive measures for *Anisakis*-induced allergy.

It was reported recently that *Anisakis* infections in rodents are similar to those in humans in terms of antibody and cytokine production [33,34]. As in human cases of gastroallergic anisakiasis [35], in the present study we observed that anti-t-Ani s 7 and anti-nAni s 7 IgE antibodies induced by live *Anisakis* larvae peaked at about day 30 p.i. and then decreased slowly over the course of 2 months (Fig. 1: t-Ani s 7 and nAni s 7). However, IgG1 antibodies were maintained at maximal values from day 20 p.i. throughout the observation period (Fig. 2: t-Ani s 7 and nAni s 7). This contrasted with our previous observations that anti-t-Ani s 7 antibodies are mainly of the IgE class in humans, and that IgE levels often remain high for several years (unpublished results). This may reflect a different antibody response to t-Ani s 7 in humans, but more probably indicates that most positive human cases of anisakiasis are in fact reinfections.

In summary, we conclude that the t-Ani s 7 polypeptide fragment of the Ani s 7 major *Anisakis* allergen is genus-specific and that IgE antibodies against this polypeptide can be induced only when the living larva inoculates it into host tissues. Consequently, the specific detection of IgE antibodies against t-Ani s 7 appears to be an excellent way to differentiate IgE antibodies induced in true *Anisakis* infections from IgE antibodies induced by other common allergens such as those present in cockroaches, chironomids and dust-mites, which may display cross-reactivity with *Anisakis* antigens such as, for example, paramyosin (Ani s 2) and tropomyosin (Ani s 3) [18,19,36]. These aspects may be of interest not only for the specific serodiagnostic of human anisakiosis but also to enable investigation of which *Anisakis* antigens are clinically relevant, and whether or not there exist other mechanisms of sensitization, apart from infection, in *Anisakis*-induced allergy.

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Disclosure

All authors declare that there are no conflicts of interest.

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