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Haemoglobin, a new major allergen of *Anisakis simplex*

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ABSTRACT

Gastro-allergic anisakiasis and *Anisakis* sensitisation associated chronic urticaria are diseases which differ in their IgE and IgG4 responses against both crude extract and specific allergens. *Anisakis* and *Ascaris* are closely related nematodes that usually cause problems with specificity in immunodiagnostics. In this study we measured IgE and IgG4 antibodies against *Anisakis simplex* sensu lato (s. l.) and *Ascaris suum* haemoglobins in sera of 21 gastro-allergic anisakiasis and 23 chronic urticaria patients. We used a capture ELISA with the anti-*Anisakis* haemoglobin monoclonal antibody 4E8g, which also recognises *Ascaris* haemoglobin. In addition, we determined specific IgE and IgG4 to both nematodes by indirect ELISA and immunoblotting. Anti-*A. simplex* s. l. haemoglobin IgE and IgG4 levels were higher in gastro-allergic anisakiasis than in chronic urticaria patients ($P = 0.002$ and 0.026, respectively). Surprisingly, no patient had detectable IgE levels against *A. suum* haemoglobin. Finally, we carried out an in silico study of the B-cell epitopes of both haemoglobin molecules. Five epitopes were predicted in *Anisakis pegreffii* and four in *A. suum* haemoglobin. The epitope propensity values of *Anisakis* haemoglobin in the equivalent IgE binding region of the allergenic haemoglobin *Chi t 1* from *Chironomus thummi*, were higher those of the *Ascaris* haemoglobin. In conclusion, we describe *A. simplex* haemoglobin as a new major allergen (*Ani s 13*), being recognised by a large number (64.3%) of sensitised patients and up to 80.9% in patients with gastro-allergic anisakiasis. The presence of a specific epitope and the different values of epitope propensity between *Anisakis* and *Ascaris* haemoglobin could explain the lack of cross-reactivity between the two molecules. The absence of IgE reactivity to *Ascaris* haemoglobin in *Anisakis* patients makes *Anisakis* haemoglobin (*Ani s 13*) a potential candidate for developing more specific diagnosis tools.

1. Introduction

Most of the parasitic anisakid nematodes use cetaceans and pinnipeds as definitive hosts, crustaceans as intermediate hosts and fish as paratenic hosts (Mattiucci and Nascetti, 2008). Live *Anisakis* L3 are able to infect humans, causing a disease known as anisakiasis (Nieuwenhuizen and Lopata, 2013). Gastro-allergic anisakiasis (GAA) was described in 2000 as a concept in which an acute allergic reaction (ranging from urticaria or angioedema to anaphylaxis) accompanies the penetration of the fish-nematode *Anisakis simplex* through the gastric mucosa (Daschner et al., 2000). The allergic reaction in GAA is simultaneous with the entrance of the nematode into the gastric mucosa. GAA, considered to be a secondary infection, gives rise to a polyclonal stimulation of all immunoglobulin isotypes including Th1 and Th2 associated IgE, IgG, IgG4 and IgA, and in addition stimulates the production of new IgE (Daschner et al., 2002).

Haemoglobins are ubiquitous proteins, present in prokaryotes, fungi, plants and animals (Hardison, 1996). In comparison with the exhaustively studied vertebrate tetrameric human haemoglobin (64 kDa) and monomeric myoglobin (17 kDa), invertebrate haemoglobins show a wide variety of functions and structures (Weber and Vinogradov, 2001).

Although sensitisation to pork and chicken haemoglobins has been reported in a case of occupational allergy (Hilger et al., 2010), only haemoglobin of the midge *Chironomus thummi* has been characterised as an allergen in humans (Baur et al., 1982). These insects may cause severe allergic respiratory diseases in sensitised subjects (Yong et al., 1999).

Nematode haemoglobin has been investigated for its high oxygen binding affinity (De Baere et al., 1992) but nothing is

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known about its allergenicity. There is evidence of cross-reactivity between arthropods and nematodes but the role of haemoglobin is still not clear. In 1997 Pascual et al. showed cross-reacting allergens among *Chironomus*, German cockroach and *Anisakis*. These allergens were identified by immunoblotting inhibition as bands between 30 and 40 kDa (Pascual et al., 1997).

Several structures of haemoglobin are available on the www.pdb.org website. The structure of *C. thummi* haemoglobin was determined in 1979 by Steigemann and Weber (PDB: 1ECA/1ECD/1ECN/1ECO). A human IgE epitope was identified in this haemoglobin (allergen *Chi t 1.01*) in the amino acid positions 80–100 by using sera from 15 allergic patients (van Kampen et al., 2001).

Regarding nematode haemoglobins, only the crystal structure of one of the two domains of *Ascaris* haemoglobin has been elucidated (PDB: 1ASH) (Yang et al., 1995). The quaternary structure was studied by electron microscopy and showed that this molecule exists as an octamer (Darawshe and Daniel, 1991; De Baere et al., 1992; Minning and Goldberg, 1998). To date, the structure of *Anisakis* haemoglobin is unknown, although the majority of the mature amino acidic sequence has been determined (Nieuwenhuizen et al., 2013).

Finally, *Anisakis* sensitisation associated chronic urticaria (CU+) is an *Anisakis*-associated allergic disease phenotype in which patients present at least twice each week with spontaneously occurring wheals, for a period of at least 6 weeks. They frequently have a previous history of fish-induced allergic and/or abdominal symptoms, together with a positive Skin Prick Test (SPT) against *A. simplex* and detectable specific IgE against *A. simplex* (Daschner et al., 2010).

GAA and CU+ differ with respect to IgE and IgG4 antibodies against crude extract and recombinant allergens such as *Ani s 1* and *Ani s 7*. A high proportion of both CU+ and GAA patients show IgE binding to *Ani s 7*, whereas IgE recognition of *Ani s 1* is significantly lower in CU+ patients. We have also observed that levels of specific IgG4 to *Anisakis* crude extract, as well as IgG4 to *Ani s 1* and *Ani s 7* recombinant allergens, were significantly higher in GAA (Cuéllar et al., 2012). Therefore, patients with different *Anisakis* sensitisation associated allergic disorders have different antigen recognition profiles.

In this study we measured IgE and IgG4 antibodies against *Anisakis* and *Ascaris* haemoglobins in sera from patients with *Anisakis* sensitisation associated with either GAA or CU+. In addition, we analysed the possible B-cell epitopes on three-dimensional models of both proteins in silico.

2. Materials and methods

2.1. Patients and serum samples

Two groups of subjects were studied and compared: 21 patients with GAA and 23 patients with CU+ were recruited prospectively during the same time-frame. GAA was diagnosed when a typical history (acute urticaria/angioedema or anaphylaxis of less than 48 h duration within 48 h of raw or undercooked-fish intake) was accompanied by further positive SPT and specific IgE against *Anisakis*. The SPT was performed with *A. simplex* extract (ALK-Abelló, Madrid, Spain) using a standard technique and was considered positive with a mean wheal diameter of 3 mm or more. Histamine at 1% concentration and saline solution (0.9%, NaCl) were positive and negative controls, respectively. CU+ patients were included if recurrent wheals were present at least twice weekly for at least 6 weeks and they displayed a positive SPT as well as specific IgE against *Anisakis* >0.35 kU/L. Patients were excluded from this study if physical stimuli were the main eliciting agents of the urticarial reaction. Written consent was obtained from all patients studied. The project was approved by the Ethics Committee of the University Hospital La Princesa, Madrid, Spain.

2.2. Routine laboratory determinations

Total IgE was analysed in all patients by ImmunoCAP® (Phadia, Uppsala, Sweden). Anti-*Anisakis*/*Ascaris* specific IgE and anti-*Anisakis* IgG4 were analysed by ImmunoCAP® (Phadia) against crude extract antigens.

2.3. Crude extract preparation

Anisakis simplex sensu lato (s. l.) L3 and *Ascaris suum* adults were homogenised in PBS and sonicated six times for 10 s. The homogenate was extracted in PBS at 4 °C overnight and subsequently delipidised with *n*-hexane. After centrifugation at 6700*g* at 4 °C, the supernatant was dialysed overnight in PBS at 4 °C (Medicell Intl. Ltd., London, UK). The protein content was quantified by the Bradford protein assay (BioRad, München, Germany).

2.4. Anti-Anisakis/Ascaris haemoglobin IgE or IgG4 determination by antigen-capture ELISA

The levels of IgE or IgG4 antibodies to haemoglobin of *A. simplex* s. l. were determined by an antigen-capture ELISA using the monoclonal antibody (mAb) 4E8g previously prepared against haemoglobin of the nematode *Anisakis pegreffii*. This mAb recognises highly immunogenic excretory-secretory haemoglobins of both *Anisakis* and *Ascaris* (Nieuwenhuizen et al., 2013). For the IgE determination, 100 µl of PBS containing 1.5 µg of the mAb 4E8g were added to each well of a 96-well ELISA plate (Costar, Corning, NY, USA) and incubated overnight at 4 °C. After washing, all wells were incubated with 200 µl of 1% BSA diluted in PBS for 1 h at 37 °C. Plates were then washed and incubated overnight at 4 °C with 100 µl of PTB (PBS plus 0.05% Tween 20 and 0.1% BSA) containing 500 µg of total protein/well from nematode crude extracts (*A. simplex* s. l. L3 or *A. suum* adult). Corresponding wells were incubated with PTB but without the extracts as control/absorbance correction. After a washing step and the addition of 100 µl/well of human sera (diluted at 1/2 with PTB), plates were incubated overnight at 4 °C. The plates were then washed repeatedly and 100 µl of a mouse anti-human-IgE mAb labelled with horseradish peroxidase (HRP) (Clone B3102E8) (SouthernBiotech, Birmingham, AL, USA) were added. After incubation at 37 °C and subsequent washing, bound Ig-HRP was detected by incubation with o-phenylenediamine (OPD; Sigma–Aldrich, Germany) in phosphate-citrate buffer (pH 5.0) with 0.04% hydrogen peroxide. The reaction was stopped with 3 N sulphuric acid. The O.D. at 490 nm was calculated by subtracting the O.D. values of the same serum in the absence of antigen. Experiments were done in duplicate.

In the case of the IgG4 measurement, the sera were diluted at 1/100 with PTB and a mouse anti-human IgG4-HRP mAb (Clone HP6025) (SouthernBiotech) was used.

For data analyses, sera were considered positive if the O.D. was more than X + 2SD, where X and SD are the corresponding mean O.D. and the S.D. obtained with 14 *Anisakis* negative control sera.

2.5. Immunoblotting

SDS–PAGE was carried out as described by Laemmli (1970) and revised by Hames (1986) using a Mini Protean® III cell (BioRad). The gels consisted of a 4% stacking gel and 12% separating gel. Worm crude extracts (25 µg/lane) were dissolved in a sample buffer (50 mM Tris–HCl buffer, pH 8.6, containing 2% SDS, 20% glycerol and 0.02% bromophenol blue) diluted 1:1 in Tris–glycine electrode buffer (25 mM Tris, 192 mM glycine, pH 8.3). Samples were loaded