Humoral immune responses induced by *Gymnorhynchus gigas* extracts in BALB/c mice

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Abstract

The aim of this study was to determine if the plerocercoid larvae of *Gymnorhynchus gigas*, a common cestode of the ray's bream (*Brama raii*), possess antigenic compounds potentially capable of provoking anaphylactic episodes. A murine experimental model, using BALB/c mice, was developed to study the humoral immune response induced by *G. gigas* extracts. A highly specific humoral immune response was detected and cross-reactions were not observed between parasite and host antigens. The presence of IgM and IgG3 levels suggest the presence of thymus-independent antigens in the parasitic extract. The IgG antibody class showed the highest levels, with the IgG1 the predominant subclass. These IgG1 levels are in accordance with the supposed presence of a type I allergic reaction after the ingestion of *G. gigas* plerocercoids parasitizing fish, as well as inducing anaphylaxia in fish. These results indicate that somatic products released from ingested larvae of *G. gigas* could induce the development of a Th2 response capable of causing allergic disorders.

Gymnorhynchus gigas Cuvier, 1817 is a Trypanorhyncha cestode characterized by ear-like appendages on their scolex, with very long, hook-bearing proboscides or tentacles that can be invaginated into proboscis bulbs. This species, which belongs to the genus *Gymnorhynchus* Rudolphi, 1819 and the family Gymnorhynchidae Dollfus, 1935, has a life-cycle involving at least two hosts. Fish may serve as final or intermediate hosts for these cestodes, which are oviparous and eggs are passed in the faeces of the final host, hatching in water to release free-swimming larvae. In the order Trypanorhyncha these larvae are known as a coracidia, which must be eaten by a suitable invertebrate intermediate host, usually a planktonic copepod. The larval cestode penetrates through the gut wall of the host and undergoes further development in the body cavity to the stage of procercoid, which is capable of infecting the fish host. If the procercoid is ingested by a suitable fish host, it penetrates the gut wall and encysts in the viscera or musculature where it develops to the plerocercoid stage. Fish in which a plerocercoid stage is formed act as second intermediate hosts. Serious economic losses occur due to mass infections of fish muscles by plerocercoids of Trypanorhyncha. The larvae are located near the vertebral column and individuals reach up to 20 cm in length and may simulate mass infection if the worm is cut several times during the process of filleting the fish. The life-cycle is completed if an infected fish is eaten by a suitable final host, in the intestine of which the cestode develops to maturity. Plerocercoids from tropical and subtropical marine fish mainly belong to the Trypanorhyncha, which have elasmobranchs as final hosts. Elasmobranchs, the rays and the sharks, occur with greater variety and in larger numbers in the tropics and subtropics than in cold waters (Möller & Andres, 1986).

Gymnorhynchus gigas plerocercoids are often found in the musculature of the ray's bream (*Brama raii*) causing a mass invasion in the muscles of fish and the mode of infection appears to be either through the skin of the fish or by ingestion of parasitized zooplankton or crustaceans (Pellegrini *et al.*, 1984).

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Since Kasuya *et al*. (1990) observed that larvae of *Anisakis* were the real causative agents in some patients with urticaria but without abdominal pain and, therefore, with no clinical suspicion of gastric/intestinal anisakidosis, many cases of allergy due to this nematode parasitizing fish have been described. Moreover, the first case of periodic anaphylactic episodes to *Anisakis simplex* in Spain was reported by Audícana *et al*. (1995), being confirmed by the prick method and specific IgE detection. In a collaborative study carried out with the Allergy and Clinical Immunology Service (Hospital del Aire, Madrid), *A. simplex* was found to be the main cause of acute recidivant urticaria in patients who usually eat fish and are not sensitized to it (Montoro *et al.*, 1997). Since Spanish people eat 78.2 g of fish (0.48 g of ray's bream) per person per day (ENNA, 1995), sea food allergens seem to have an important role in the development of anaphylactic episodes in our country.

The aim of the present study was to determine if plerocercoid larvae of *G. gigas*, a common cestode of fish, possess antigenic compounds potentially capable of provoking anaphylactic episodes. For this reason, we have developed a murine experimental model, using BALB/c mice, to compare the humoral immune response induced by *G. gigas* extracts with those obtained in our experimental conditions after immunization of the same animals by the injection of *A. simplex* extracts.

Materials and methods

Parasites

Gymnorhynchus gigas larvae were collected individually from the muscle of naturally infected ray's bream (*Brama raii*) and thoroughly washed in water. Hosts were obtained from a fish supplier in Madrid, Spain.

Preparation of crude extracts from B. raii *and* G. gigas

Crude extracts of *G. gigas* were obtained using a modification of the Welch *et al*. (1983) method by homogenization and extraction in PBS at 4°C overnight instead of ultrasonic disruption (Cuéllar *et al*., 1992). The same method was using to obtain crude extracts from the muscle of *B. raii*.

Mice

Ten BALB/c mice were immunized with crude *B. raii* or *G. gigas* extracts either as multiple doses of 1 ml of antigens in FCA (1 mg of protein ml^{-1} in final volume) given weekly for 3 weeks by the intramuscular route.

Serum

Animals were bled weekly under ether anaesthesia, by the retroorbital plexus from 3 to 14 weeks post-immunization (p.i.). After bleeding, blood samples from each group of mice were pooled and centrifuged to obtain sera.

Specific antibody levels

Specific antibody levels were measured by ELISA. The 96-well microtitre plates (Nunc-Immuno Plate Poly Sorp™) were coated overnight by the addition of 1 μg per well of *B. raii* or *G. gigas* antigen diluted in a carbonated buffer to 0.1 M at pH 9.6 at 4°C. Several wells were kept uncoated as control for nonspecific reactions (Cuéllar *et al*., 1992). After washing three times with 0.05% PBS-Tween 20 (PBS-Tween), blocking was carried out by adding 250 μl per well of 0.1% BSA in PBS, incubating for 1 h at 37°C. After washing, 100 μl of serum samples were diluted in 1/100 in PBS-Tween, 0.1% BSA added in duplicate and the samples incubated at 37°C for 2 h. As negative controls, sera from non-infected mice were used (all mice were bled at day 0). Once the plates were washed, 100 μl per well of a goat affinity isolated, horseradish peroxidase conjugated antibody specific to mouse IgG + IgM (H + L), IgM (μ), IgG (γ), IgG1(γ1), IgG2a (γ2a), IgG2b (γ2b), IgG3 (γ3) and IgA (α) (CALTAG LABORATORIES, San Francisco, California), at the appropriate dilution in PBS-Tween, 0.1% BSA, were added and incubated for 1 h at 37°C. After washing, 100 μl per well of substrate (O-phenylene-diamine; SIGMA) were added at 0.04% in a 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. Results were expressed as O.D.p–O.D.c indexes by subtracting the mean O.D. of the control from the mean O.D. of the test sera once the non-specific reaction with the BSA used in the blocking was subtracted (Cuéllar *et al*., 1997).

Statistical analysis

Data were analysed with SPSS/PC software. Statistical comparisons were done with the ANOVA one-way test. Multiple comparisons post-hoc were realized with the Bonferroni methods. P values < 0.05 were considered to be statistically significant.

Results and discussion

The aim of this study was to determine the presence of antigenic compounds potentially capable of provoking anaphylactic episodes in the plerocercoid larvae of *G. gigas*, a common cestode of the ray's bream. The immunization procedure and mouse strain were selected because of their ability to elicit strong responses to crude extracts of several helminth parasites (Cuéllar *et al*., 1992, 1995). In particular, high and long-term humoral responses against the crude extract of *A. simplex* have been previously observed in similar experimental conditions (Cuéllar *et al*., 1997).

Both immunizations, with *B. raii* or *G. gigas* extracts, produced highly specific humoral immune responses. Cross-reactions between both antigens were not observed (fig. 1), suggesting that both *B. raii* and *G. gigas* extracts could be useful in skin tests by the prick method to investigate if the ray's bream-induced urticaria is an allergic response to plerocercoid antigen rather than to the fish itself.

The production of the IgM isotype was lower, being practically undetectable in mice immunized with the *B. raii* extract (fig. 2A). However, after the injection of *G. gigas* antigen, antibody levels were obtained, remaining at detectable values until end of the experiment

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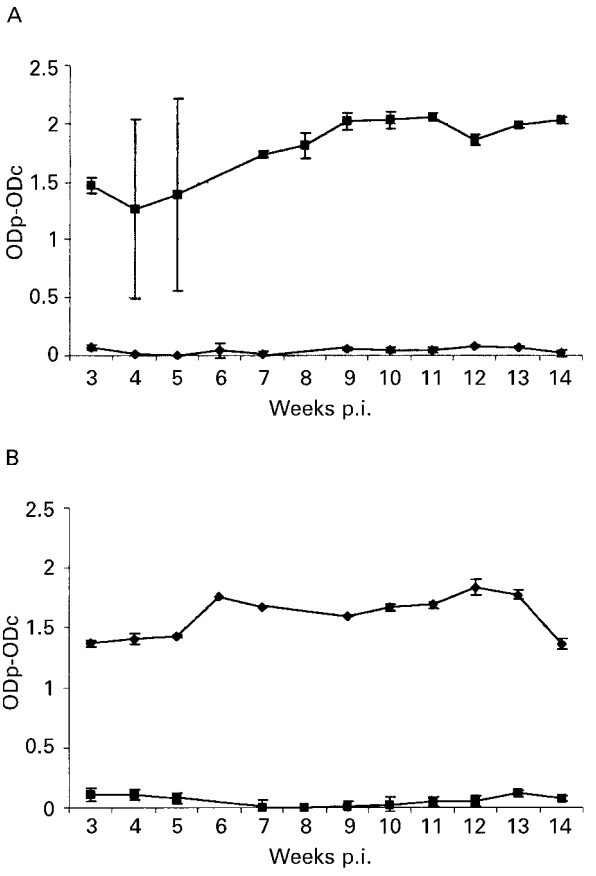

Fig. 1. Dynamics of IgG+IgM production in BALB/c mice immunized with *Brama raii* (■) or *Gymnorhynchus gigas* (◆) extract, against *B. raii* (A) and *G. gigas* (B) antigens. Standard errors are included.

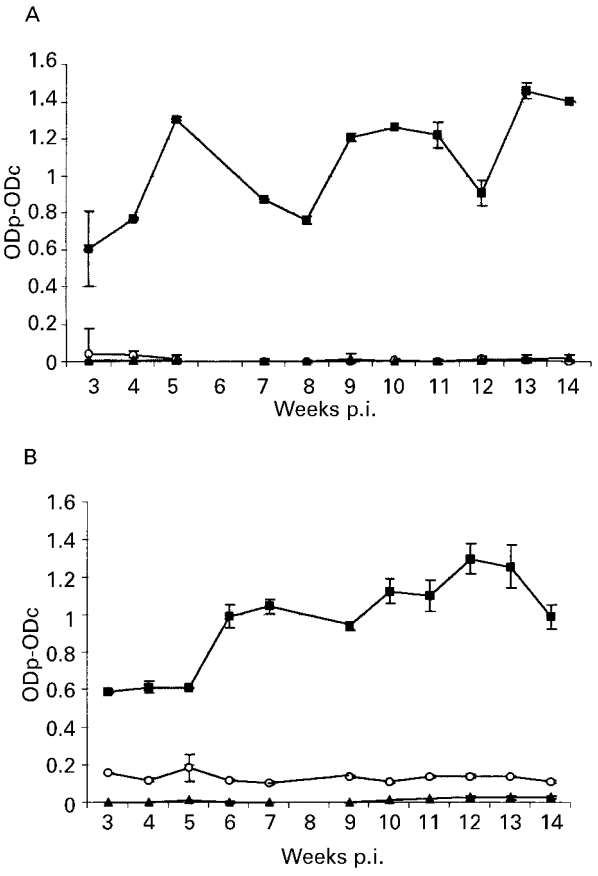

Fig. 2. Dynamics of specific IgM (○), IgG (■) and IgA (▲) production in BALB/c mice immunized with *Brama raii* (A) or *Gymnorhynchus gigas* (B) extracts. Standard errors are included.

(fig. 2B). This result suggests that thymus-independent antigens could be present in the *G. gigas* extract.

Conversely to the results obtained with the IgM isotype, the IgG antibody class showed much higher levels against both antigens (fig. 2), with IgG1 being the predominant subclass (fig. 3). IgG1 secretion is enhanced by IL-4 (Snapper *et al.*, 1988), factor secreted by the T-cell subset termed the Th2 cell (Mosmann *et al.*, 1986). These IgG1 levels are in accordance with the supposed presence of a type I allergic reaction after the ingestion of *G. gigas* plerocercoids parasitizing fish as well as the induction of anaphylaxia in fish. On the other hand, human IgG4 may represent the biological counterpart of murine IgG1 (Flores-Romo *et al.*, 1990; Snapper & Mond 1993). The balance between antigen-specific IgG4 and IgE (under IL-4 regulation) has been reported to be important in the regulation of other antigen-specific immediated hypersensitivities (Mahanty *et al.*, 1994). The present results might indicate that *G. gigas* somatic products released from ingested larvae could induce the development of a Th2 response when larvae are ingested by fish.

The levels of the IgG2a isotype were detectable with sera from mice immunized with both *B. raii* and *G. gigas* antigens (fig. 3), although with higher levels following the *B. raii* injection (fig. 3A). The presence of IgG1 and IgG2a isotypes would suggest that both Th1 and Th2 cells are active, although with a Th2-dominant response after the *G. gigas* injection. In these mice, the IgG2a production, the secretion of which is enhanced by IFN-γ (Th1-dependent) (Snapper *et al.*, 1988), was lower than that observed in mice immunized with the *B. raii* extract. In the case of *A.simplex* immunization, both Th1 and Th2 cells are active, although with a Th2-dominant response during the early stage (Cuéllar *et al.*, 1997).

On the other hand, similar levels of the IgG2b isotype were detected in both groups, remaining at detectable levels, although fluctuations were present until end of the experiment (fig. 3). TGF-β selectively promotes the class switch to IgG2b (Snapper & Mond, 1993). This cytokine, produced by macrophages would serve to limit the Th1-mediated effector function (Oswald *et al.*, 1992). The T-cell subset is apparently activated, as shown by the levels of IgG2a detected.

The BALB/c mice immunized with the *G. gigas* extract showed detectable levels of IgG3 (fig. 3B), but these were

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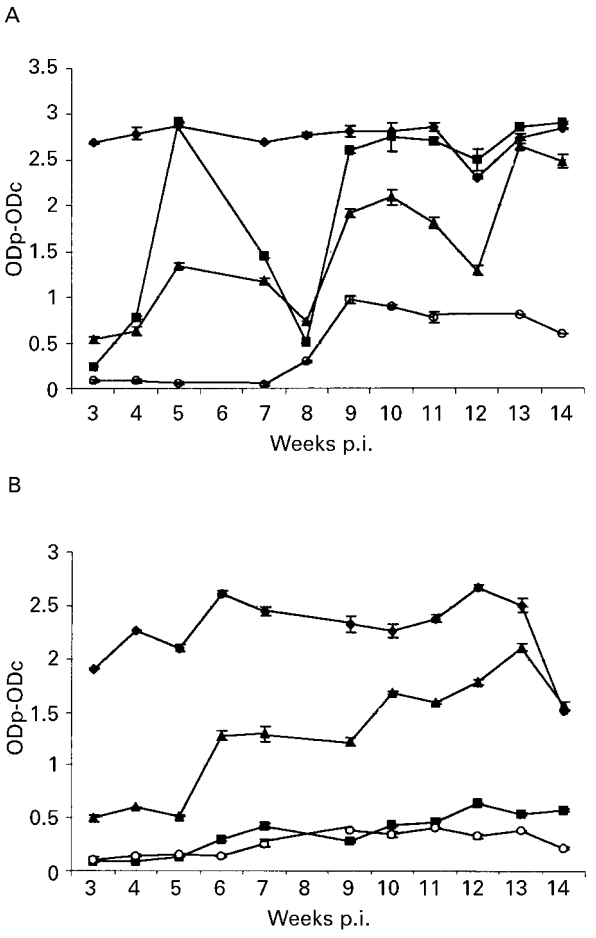

Fig. 3. Dynamics of specific IgG1 (◆), IgG2a (■), IgG2b (▲) and IgG3 (○) production in BALB/c mice immunized with *Brama raii* (A) or *Gymnorhynchus gigas* (B) extracts. Standard errors are included.

lower than those observed following the *B. raii* extract injection (fig. 3A). This response, observed after 6–7 weeks p.i., could be due to T-independent polysaccharide antigens. These T-independent-2 antigens might bind to a cell-surface molecule common to murine cells and lead to isotype switching with specific IgG3 production (Janeway & Travers, 1994).

Statistical analyses were therefore carried out with the four IgG subclasses tested. When specific antibodies against *B. raii* antigen were tested, IgG1 was not statistically different to IgG2a by weeks 5, 10 and 14 p.i. In the case of the *G. gigas* extract, differences were neither observed after 3 and 5 weeks p.i. when IgG2a and IgG3 were tested nor with IgG1 and IgG2b by week 14 p.i. All the other groups analysed were statistically different to each other, thus confirming the validity of the observed differences.

IgA responses were undetectable following both *B. raii* and *G. gigas* immunizations, probably due to an immunization procedure incapable of promoting this response (fig. 2).

Although complementary clinical trials on human patients are required, the results from this preliminary study indicate that *G. gigas* antigens might be capable of causing allergic disorders in patients who usually eat fish and are not sensitized to it. Moreover, in experimental animals, extracts of these cestodes induce acute, sub-acute or even chronic symptoms, such as enteritis, hypotension, nervousness and congestion, all of which are mitigated by the administration of antihistaminic drugs (Minniti *et al.*, 1995).

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