Humoral Immune Responses Induced by *Kudoa* sp. (Myxosporea: Multivalvulida) Antigens in BALB/c Mice

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The majority of Kudoa *species infect the somatic muscle of fish establishing cysts. As there is no effective method to detect infected fish without destroying them these parasited fish reach the consumer. This work was developed to determine whether this parasite contains antigenic compounds capable of provoking an immune response in laboratory animals, in order to consider the possible immunopathological effects in man by the ingestion of* Kudoa *infected fish. BALB/c mice were injected by the subcutaneous route with the following extracts suspended in aluminium hydroxide: group 1 (black* Kudoa *sp. pseudocyst extract), group 2 (white* Kudoa *sp. pseudocyst extract), and group 3 (non-infected hake meat extract). Specific antibody levels were measured by ELISA against homologous and heterologous antigens. The highest responses were obtained from the black* Kudoa *sp. pseudocyst extract (group 1). The low optic density levels detected in group 3 proved that the results obtained in groups 1 and 2 were a consequence of the parasitic extract injection. The IgG1 was the predominant subclass. IgE detected in groups 1 and 2 showed the possible allergenic nature of some of the components of the parasitic extract. High IgA levels and medium IgG2a and IgG3 levels were obtained in groups 1 and 2. Low IgG2b responses were shown. No cross-reactions between* Kudoa *sp. pseudocyst extracts and the non-infected hake meat extract were observed.*

Key words: *Kudoa* - Myxosporea - pseudocyst - immunoglobulins - BALB/c

The majority of *Kudoa* species infect the somatic muscle of marine and estuarine fish establishing cysts, which contain many spores. As the parasite grows, it produces proteolytic enzymes (Patashnik et al. 1982, Tsuyuki et al. 1982) that break down the filaments of the muscle fibre (Stehr & Whitaker 1986). While the parasite is within a muscle fibre, it is undetected by the host's immunological system. It is during this stage when the parasite contains many developing and mature spores, that the infected fibres have a white appearance. As the parasite grows, it breaks the sarcolemma and the host recognizes the presence of the parasite (Moran et al. 1999). Then, there is a rapid development of a fibroblast layer around the parasite (Morado & Sparks 1986, Stehr & Whitaker 1986) and the cyst, more properly, pseudocyst, quickly acquires a black appearance. However, the process of resorption is slower than that of the development of pseudocysts. Consequently, the net effect is an accumulation of black pseudocysts as the infection progresses (Kabata & Whitaker 1986). In contrast, no cellular response is seen in "newer" hosts, where pseudocysts can completely fill muscle fibres and rupture them, releasing spores and other parasitic materials.

In recent years, private fish farming of salmonids has become an important industry in several countries. Until 1990, salmonid infections by genus *Kudoa* were unusual. However, the increment in the number of cases has been spectacular: British Columbia (Whitaker & Kent 1991), Spain (Barja & Toranzo 1993), France (Holliman 1994), Ireland (Palmer 1994) and Scotland. The smoking process, at 50ºC for about 10 h, allows proteolytic enzymes produced by the parasite to lysate some areas of the fillets, resulting a poor-quality flesh.

There is no doubt in the extraordinary development of genus *Kudoa* in the last years and its notable consequences. Considering that there is not any effective method to detect parasited fish without destroying them, it does not result strange that infected fish reach the consumer. Despite the black or white appearance of pseudocysts in fish meat, they are frequently unnoticed and not always the myoliquefaction process is so intense.

In Spain, the consumption of imported Chilean hake *Merluccius gayi gayi* (Guichenot 1848) is higher than the consumption of hake proceeding from the Cantabric Sea, which has a better quality but also a higher price. *Kudoa* infected fish have been lately detected in this imported hake, which is consumed not only fresh but also frozen. Consequently, we have developed this work to determine whether this parasite contains antigenic compounds capable of provoking an immune response in laboratory animals, in order to consider the possible immunopathological effects in man by the ingestion of *Kudoa* infected fish.

MATERIALS AND METHODS

Kudoa sp. pseudocysts - *Kudoa* sp. pseudocysts were manually obtained from the skeletal musculature of Chilean hakes *M. gayi gayi* destined for human consumption. Pseudocysts were carefully separated from any interfering fish tissue. Afterwards, they were classified as black pseudocysts and white pseudocysts by observing their content by light microscope and then finally frozen at

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-20ºC until used. Pseudocysts with intermediate characteristics were discarded.

Extracts - Both *Kudoa* sp. pseudocyst forms and non-infected hake meat were individually homogenized in a hand-operated glass tissue grinder in PBS at 4ºC. In order to release spore contents, *Kudoa* sp. homogenates were frozen at -80ºC and lately sonicated by 20 pulses of 10 sec with a Virsonic 5 (Virtis, NY, USA) set at 70% output power, in a ice-water bath. All the homogenates were extracted in PBS at 4ºC overnight. The hake meat homogenate was subsequently delipidized with n-hexane and centrifuged as the *Kudoa* sp. homogenates at 8,497 g for 30 min at 4ºC (Biofuge 17RS: Heraeus Sephatech, GmbH, Osterode, Germany). The supernatants were dialysed overnight at 4ºC in PBS. Protein content of the extracts was estimated by the Bradford method (1976) and the extracts were frozen at -20ºC until used.

Antigen coupling - Black and white *Kudoa* sp. extract fractions were used for coupling the antigens to a carrier protein. Pierce's Imject® PharmaLink™ Inmunogen Kit was employed, which use a cationized bovine serum albumin (cBSA) as a carrier (SuperCarrier® Immune Modulator), a BSA in which the carboxylic groups are modified to produce a protein with a basic pI. The enhanced immunogenic properties of cBSA are imparted to the molecule covalently coupled to it. Cationization of an antigen provides the benefit of increased immunogenicity. Coupling process was done via the Mannich reaction, according to the manufacturer's instructions.

Animals and immunization protocol - Thirty BALB/c mice were divided into five equal groups and injected by the subcutaneous route with the obtained extracts suspended in aluminium hydroxide (Imject® Alum, Pierce) as an adjuvant: group 1 (immunization with 100 µg/mouse of native black *Kudoa* sp. pseudocyst extract), group 2 (immunization with 100 µg/mouse of native white *Kudoa* sp. pseudocyst extract), group 3 (immunization with 100 µg/mouse of non-infected hake meat extract), group 4 (immunization with 50 µg/mouse of black *Kudoa* sp. pseudocyst extract coupled to cBSA) and group 5 (immunization with 50 mg/mouse of white *Kudoa* sp. pseudocyst extract coupled to cBSA). Two weeks later, they were injected again with an equal dose. Besides, a non-injected identical group was used as a control group. The quantity of carrier-coupled extracts injected per mouse was the minimum recommended by the kit protocol, while the quantity of the native extracts was in the advised range for its administration as coupled extracts. The ratio (v/v) of adjuvant to extract was 1:3.

Sera - Animals were bled weekly, including the control group, under ether anaesthesia, by the retroorbital plexus, from the fourth to the 21st week since the first immunization. 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 Polysorp™, Brand Products, Denmark) were coated overnight at 4ºC by the addition of 10 µg/ml per well of *Kudoa* sp. extracts, whether coupled or not to cBSA, and the meat hake extract, diluted in a carbonated buffer to 0.1M at pH 9.6 at 4ºC. Several wells were kept uncoated as a control for non-specific reactions, except when the plates were coated with the *Kudoa* sp. antigens coupled to the carrier; then, these wells were coated with a 10 µg/ml solution per well of cBSA (SuperCarrier® Immune Modulator, Pierce) diluted in the same carbonated buffer to measure unspecific reactions to the cBSA. After washing the plates three times with 0.05% PBS-Tween 20 (PBS-Tween), blocking was carried out by adding 200 µl per well of 0.1% BSA (Sigma, St Louis, MO, USA) in PBS, incubating for 1 h at 37ºC. After washing, 100 µl of serum samples were diluted 1/100 (1/10 when measuring IgE) in PBS-Tween, 0.1% BSA, added in quadruplicate and incubated at 37ºC for 2 h. As negative controls, sera from the control group were used. 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), IgG (γ), IgG1 (γ1), IgG2a (γ2a), IgG2b (γ2b), IgG3 (γ3), IgA (α) (Caltag Laboratories, San Francisco, California, USA), IgM (µ) (Sigma, St Louis, MO, USA) and IgE (ε) (sheep, The Binding Site), at the appropriate dilution in PBS-Tween, 0.1% BSA, were added and incubated for 1 h at 37ºC. After washing, 100 µl per well of substrate (O-phenylene-diamine; Sigma, St Louis, MO, USA) were added at 0.04% in a phosphate-citrate buffer (pH 5.0) with 0.04% hydrogen peroxide. The reaction was stopped with 3N sulphuric acid and the plates were read at 490 ηm. 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 ($O.D._p$/$O.D._c$ when measuring IgE) once the non-specific reaction with the BSA used in the blocking (and with the cBSA when the antigen was coupled to the carrier) was subtracted.

RESULTS

Dynamics of antibody responses in BALB/c mice immunized with Kudoa sp. pseudocyst extracts - The highest IgG + IgM (Fig. 1) and IgG (Fig. 2) responses were obtained from the black *Kudoa* sp. pseudocyst extract (group 1).

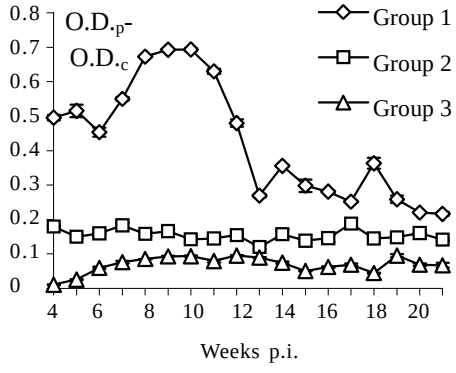

Fig. 1: dynamics of specific IgG + IgM production induced by the different extracts in each group. Group 1: immunization with 100 µg/mouse of native black *Kudoa* sp. pseudocyst extract; group 2: immunization with 100 µg/mouse of native white *Kudoa* sp. pseudocyst extract; group 3: immunization with 100 µg/mouse of non-infected hake meat extract. Standard errors are included.

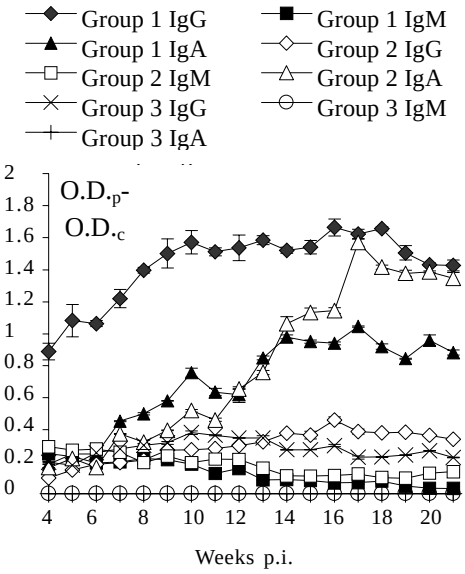

Fig. 2: dynamics of specific IgG, IgM and IgA production induced by the different extracts in each group. Group 1: immunization with 100 µg/mouse of native black *Kudoa* sp. pseudocyst extract; group 2: immunization with 100 µg/mouse of native white *Kudoa* sp. pseudocyst extract; group 3: immunization with 100 µg/mouse of non-infected hake meat extract. Standard errors are included.

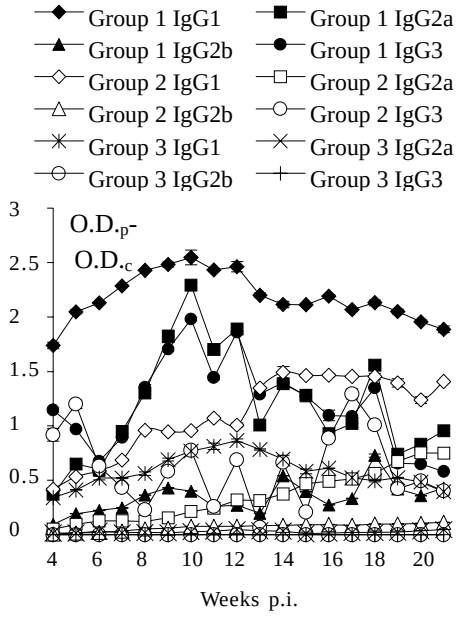

Fig. 3: dynamics of specific IgG1, IgG2a, IgG2b and IgG3 production induced by the different extracts in each group. Group 1: immunization with 100 µg/mouse of native black *Kudoa* sp. pseudocyst extract; group 2: immunization with 100 µg/mouse of native white *Kudoa* sp. pseudocyst extract; group 3: immunization with 100 µg/mouse of non-infected hake meat extract. Standard errors are included.

Low (IgG + IgM, IgG, IgG2a) or null (IgG2b, IgG3, IgM, IgA, IgE) responses were observed from the group immunized with non-infected hake meat extract (group 3; Figs 1-4). Higher IgG1 levels were obtained in this group (Fig. 3).

The low responses (data not shown) from the carrier-coupled extracts (groups 4, 5; immunized with black or white *Kudoa* sp. pseudocyst extract, respectively, coupled to cBSA) showed their lower capacity to induce a humoral response compared to the native antigens (groups 1, 2).

The high $O.D._p$-$O.D._c$ indexes observed in sera from black *Kudoa* sp. pseudocyst extract (group 1) when the production of specific IgG1, IgG2a, IgG2b and IgG3 was studied (Fig. 3), also showed its higher antigenic properties towards the white *Kudoa* sp. pseudocyst extract (group 2). The variability in the IgG3 levels was a common feature in the groups immunized with the native *Kudoa* sp. pseudocyst extracts (groups 1, 2), mostly in the group injected with the extract obtained from white pseudocysts (group 2). The IgM and IgA responses (Fig. 2) were very similar with both native parasite extracts (groups 1, 2).

The IgE production (Fig. 4) was higher in the mice injected with the black *Kudoa* sp. pseudocyst extract (group 1) than that obtained from the white pseudocyst extract (group 2); moreover, while in the latter the IgE levels decreased after reaching a maximum, in the former they kept more or less constant until the end of the experiment.

Study of possible cross-reactions between homologous and heterologous antigens - The sera obtained from the BALB/c mice immunized with the native *Kudoa* sp. pseudocyst extracts (groups 1, 2) did not react with the hake meat extract. In the same way, the sera from the mice injected with the hake meat extract (group 3) did not react with the native pseudocyst extracts. The responses induced by sera from group 2 (immunized with native white *Kudoa* sp. pseudocyst extract) against the black pseudocyst extract were higher than those obtained with the homologous extract (data not shown).

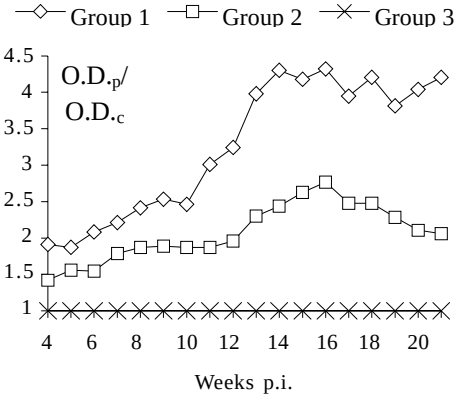

Fig. 4: dynamics of specific IgE production induced by the different extracts in each group. Group 1: immunization with 100 µg/mouse of native black *Kudoa* sp. pseudocyst extract; group 2: immunization with 100 µg/mouse of native white *Kudoa* sp. pseudocyst extract; group 3: immunization with 100 µg/mouse of non-infected hake meat extract. Standard errors are included.

DISCUSSION

It seems that, despite the low or null response that myxosporeans provoke in their natural hosts (Hallyday 1974, McArthur 1977, Siau 1980), perhaps as a consequence of an antigenic mimicry (Pauley 1974, McArthur & Sengupta 1982), these parasites can induce an antibody response in other animals. As a result of the extent of Myxosporea in the sea world, the ingestion of these parasites with the fish we usually eat is nowadays common, while their immune consequences are still unknown.

We decided to use a carrier protein (cationized BSA) as a method for inducing a prompt and more amplified murine immune response. Nevertheless, the immunization with the native extracts was not discarded. Besides, several experiments were made to check out the ELISA, coating the antigen in different concentrations and using the sera at several dilutions (results not shown). The best outcomes were a 10 µg/ml concentration for antigen-coating and a 1/100 dilution for the sera.

In a first analysis of the results it appeared that, in opposition to what was expected, the response obtained from the groups immunized with the extracts coupled to the carrier (groups 4, 5: immunized with black or white *Kudoa* sp. pseudocyst extract, respectively, coupled to the carrier) was lower than the one obtained from the native extracts (groups 1, 2: immunized with native black or white *Kudoa* sp. pseudocyst extract, respectively). Besides, the sera of the mice injected with the cBSA-coupled extracts did not react to the homologous native extracts (data not shown). This fact points out an important alteration in the antigen nature. Possibly, the antigen bound to the carrier was not properly achieved. The chemical nature of the antigen is unknown. On the other hand, it is feasible that the cBSA has disfigured or changed the three-dimensional antigen arrangement and, therefore, its processing and presentation were not suitable. Alternatively, we have checked the antigen immunogenicity in the absence of the carrier. Therefore, we can presume that the coupling of the antigenic molecules to the carrier has produced an increased amount of antigen over the optimum quantity.

The comparative study of most of the immunoglobulins clearly showed the primacy of the black pseudocyst extract levels (group 1: immunized with native black *Kudoa* sp. pseudocyst extract) towards the white ones (group 2: immunized with native white *Kudoa* sp. pseudocyst extract). At lower levels, this fact repeated itself in the homologous extracts linked to the cBSA (data not shown). It suggested that the extract obtained from black *Kudoa* sp. pseudocysts, which are featured with a higher density of degenerated mature spores (Stehr & Whitaker 1986), has a stronger ability to induce antibody responses. The low optic density levels obtained in the group inoculated with the non-infected hake meat (group 3) proved that the results obtained in the *Kudoa* sp. groups, specially in those injected with the native antigen (groups 1, 2), were a consequence of the parasitic extract injection and, in case of a possible contamination, it did not affect the final results.

The IgG1 always appeared as the predominant subclass in the different groups, reaching $O.D._p$-$O.D._c$ levels of 2.5 in the injected mice of the group 1 (immunization with native black *Kudoa* sp. pseudocyst extract). Simultaneously, IgE detected in both groups 1 and 2 (immunized with native black or white *Kudoa* sp. pseudocyst extract, respectively) showed the possible allergenic nature of some of the components of the parasitic extract. These components could be responsible for type I hypersensibility reactions after its ingestion. Besides, murine IgG1 antibodies are capable of mediating the mast cells degranulation.

Analysing the different classes and subclasses of specific immunoglobulins it can be deduced that although a clear expansion of Th2 lymphocytes exists, the medium IgG2a levels in the group 2 and high in the group 1 suggest that there are also Th1 active clones. This fact has been observed in other parasitic models, like in the BALB/c mice response to a secondary hydatidosis experimentally induced (Haralabidis et al. 1995), where in addition to a Th2 response there is a parallel Th1 expansion, which provokes an inflammatory response being a defensive mechanism of the host.

In order to study the specificity of the antigens used, sera with high response to their homologous antigens were selected (from group 1 – immunization with native black *Kudoa* sp. pseudocyst extract –, sera from the weeks 10 and 11 p.i.; group 2 – immunization with native white *Kudoa* sp. pseudocyst extract –, weeks 15 and 16 p.i.; group 3 – immunization with non-infected hake meat extract –, weeks 10 and 11 p.i.; weeks 20 and 21 p.i. of each group when measuring IgE) and confronted to their related heterologous antigens. The different studies of each immunoglobulin, indicated that the sera obtained from the BALB/c mice immunized with the native *Kudoa* sp. pseudocyst extracts (groups 1, 2) did not react with the hake meat extract. In the same way, the sera resulting from the hake meat extract injection in these mice did not react with the pseudocyst extracts. In addition, these results confirmed that the black pseudocysts have more capability to stimulate the antibody production. The antibodies induced by the white pseudocyst extract gave a higher response with the black pseudocyst extract than with the homologous extract.

Consequently, the experiments realized concerning the possible existence of cross-reactions between the *Kudoa* sp. antigens and the skeletal musculature of the Chilean hake reveal that the parasite does not share antigens with *M. gayi gayi*. The coexistence of shared antigens assumed by Pauley (1974) and McArthur and Sengupta (1982) for the genus *Myxobolus* as being responsible of the antigenic mimicry is not shown in the Chilean hake and the infecting *Kudoa* sp. Antigenic mimicry was considered by these authors as a reason for the "immune impunity" which many myxosporeans seem to have. As we explained before, the progressive darkness of the pseudocysts is a consequence of the spore degeneration produced by the cellular fish response. These pseudocysts are common in *M. gayi gayi*. There are no studies about the immune response in this fish. However, considering that this *Kudoa* species does not have, at least in the Chilean hake, the

immune privileges of other myxosporeans in some hosts, a cellular response accompanied with the antibody production would not be strange. Besides, a possible antigenic mimicry does not seem to exist.

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