SPECIFIC IgE INDUCED BY *KUDOA* SP. (MYXOSPOREA: MULTIVALVULIDA) ANTIGENS IN BALB/C MICE

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Summary:

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. 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. 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). Specific IgE levels were measured by ELISA. IgE detected in both groups 1 and 2 showed the possible allergenic nature of some of the components of the parasitic extracts.

KEY WORDS : antigen, ELISA, IgE, *Kudoa*, Myxosporea, pseudocyst

Résumé : IgE SPÉCIFIQUES INDUITES PAR LES ANTIGÈNES DE *KUDOA* SP. (MYXOSPOREA: MULTIVALVULIDA) CHEZ LA SOURIS BALB/C

La plupart des espèces de Kudoa infectent le muscle somatique des poissons en formant des kystes. Comme il n'existe pas de méthodes efficaces pour détecter les poissons infectés sans les abîmer, ces poissons parasités arrivent jusqu'au consommateur. Nous avons développé ce travail pour déterminer si ce parasite contient des composants antigéniques susceptibles de provoquer une réponse immunitaire sur des animaux de laboratoire, et pour considérer les possibles effets immunopathologiques chez l'homme de l'ingestion de poissons infectés par Kudoa. Nous avons injecté à des souris BALB/c, par voie sous-cutanée, deux types d'extraits en suspension dans de l'hydroxyde d'aluminium : groupe 1 (extraits de pseudokystes noirs de Kudoa sp.), groupe 2 (extraits de pseudokystes blancs de Kudoa sp.). Nous avons mesuré les niveaux d'IgE spécifiques par ELISA. Les niveaux d'IgE détectés dans chacun des groupes ont démontré la possible nature allergénique de quelques uns des composants des extraits parasitaires.

MOTS CLÉS : *antigen, ELISA, IgE, Kudoa, Myxosporea, pseudokyste.*

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).

Considering that there is not any effective method to detect parasitized fish without destroying them, it is not unusual that infected fish reach the consumer. Despite the black or white appearance of pseudocyst in fish meat, they are frequently unnoticed and the myoliquefaction process is not always intense, especially in the older hosts.

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 by the ingestion of *Kudoa* infected fish.

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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 white pseudocysts and black pseudocysts by observing their content by light microscope and then finally frozen at – 20° C 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 4° C. In order to release spore contents, *Kudoa* sp. homogenates were frozen at – 80° C and lately sonicated by 20 pulses of 10 s with a Virsonic 5 (Virtis, NY, USA) set at 70 % output power, in an ice-water bath. All the homogenates were extracted in PBS at 4° C 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 4° C (Biofuge 17RS: Heraeus Sephatech, GmbH, Osterode, Germany). The supernatants were dialysed overnight at 4° C in PBS. Protein content of the extracts was estimated by the Bradford method (1976) and the extracts were frozen at – 20° C until used.

Animals and immunization protocol

Eighteen BALB/c mice were divided in three equal groups and injected by the subcutaneous route with the obtained extracts suspended in aluminium hydroxide (Imject® Alum, Pierce) as adjuvant: group 1 (immunization with 100 µg/mouse of black *Kudoa* sp. pseudocyst extract), group 2 (immunization with 100 µg/mouse of white *Kudoa* sp. pseudocyst extract), group 3 (immunization with 100 µg/mouse of non-infected hake meat extract). Two weeks later, they were injected again with an equal dose. Besides, a non-injected identical group was used as a control group. 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 4° C by the addition of 10 µg/ml per well of *Kudoa* sp. antigens diluted in a carbonated buffer to 0.1M at pH 9.6 at 4° C. Several wells were kept uncoated as a control for non-specific reactions. 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 one hour at 37° C. After washing, 100 µl of serum samples were diluted 1/10 in PBS-Tween, 0.1 % BSA, added in triplicate, against their homologous antigen, and incubated at 37° C for two hours. 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 IgE (ε) (sheep, The Binding Site), at the appropriate dilution in PBS-Tween, 0.1 % BSA, were added and incubated for one hour 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 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 dividing 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.

RESULTS

The IgE production against their homologous antigens (Fig. 1) was higher in the mice injected with the black *Kudoa* sp. pseudocyst extract (Group 1) than that obtained with the white pseudocyst extract (Group 2), being the differences between both groups statistically significant in all the weeks ($p < 0.05$);

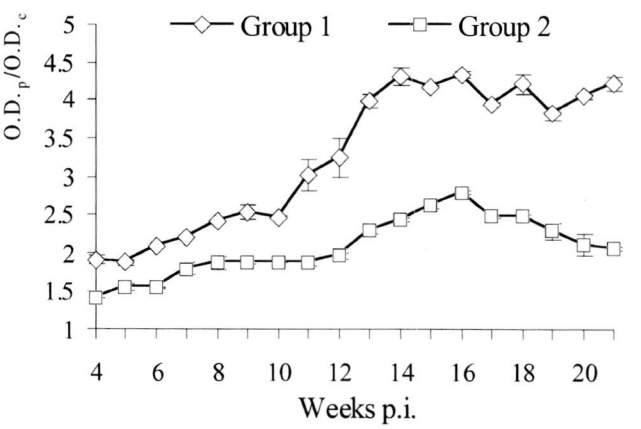

Fig. 1. – Dynamics of specific IgE production induced by the *Kudoa* sp. extracts in BALB/c mice against its homologous antigen. Group 1: immunization with 100 µg/mouse of black *Kudoa* sp. pseudocyst extract. Group 2: immunization with 100 µg/mouse of white *Kudoa* sp. pseudocyst extract. Standard errors are included.

moreover, while in the later the IgE levels decreased after reaching a maximum, in the former they kept more or less constant until the end of the experiment. No IgE response was detected in the group immunised with the non-infected hake meat extract (Group 3).

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 (Muñoz *et al.*, 2000; Chase *et al.*, 2001). 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. Consequently, IgE detected in both groups 1 and 2 (immunized with black or white *Kudoa* sp. pseudocyst extract, respectively) showed the possible allergenic nature of some of the components of the parasitic extracts. These components could be responsible for type I hypersensitivity reactions after their ingestion. The absence of IgE response in the group inoculated with the non-infected hake meat (Group 3) proved that the results obtained in the *Kudoa* sp. groups (Groups 1 and 2), were a consequence of the parasitic extract injection and, in case of a possible contamination, it did not affect the results. The IgE production was higher in the mice injected with the black *Kudoa* sp. pseudocyst extract than that obtained with the white pseudocyst extract. 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. These pseudocysts were the most frequently found in the Chilean hakes.

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