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The effect of lectins on the attachment and invasion of *Enteromyxum scophthalmi* (Myxozoa) in turbot (*Psetta maxima* L.) intestinal epithelium *in vitro*

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Abbreviations

Con A: *Canavalia ensiformis*; SBA: *Glycine max*; PBS: phosphate buffer saline; PSA: antibiotic/antimycotic mixture; FBS: foetal bovin serum; EDTA: ethylenediaminetetraacetic acid; DTT: dithiothreitol; HBSS: Hanks' Balanced Salt Solution; Man: mannose; Glc: glucose; GlcNAc: Nacetylglucosamine; GalNAc: Nacetylgalactosamine; Gal: galactose; MBL: mannose-binding lectin.

Abstract

The involvement of the lectin/carbohydrate interaction in the invasion of the turbot intestinal epithelium by *Enteromyxum scophthalmi* was studied *in vitro* using explants of turbot intestine and pre-treatment of parasite stages with the plant lectins of *Canavalia ensiformis* (Con A) and *Glycine max* (SBA). Both lectins inhibited the attachment and invasion of *E. scophthalmi* stages to the intestinal epithelium, though the inhibitory effect was higher for SBA than for Con A. Such results point to the involvement of N-acetyl-galactosamine (GalNAc) and galactose (Gal) residues and also of mannose/glucose residues in the *E. scophthalmi*-intestinal epithelium interaction. The inhibitory effect of both lectins on the parasite adhesion and penetration points to the interest of further studies to confirm the presence of putative lectins recognising GalNAc-Gal and mannose/glucose residues in turbot intestine. The obtained results demonstrated also the adequacy of turbot intestinal explants as an *in vitro* model to study the interaction with *E. scophthalmi*.

Keywords: Myxosporea, Teleostei, *Scophthalmus*, explants, ConA lectin, SBA lectin

1. Introduction

The phylum Myxozoa includes a large number of species most of them parasites of fish. Some myxozoans cause disease and impact upon wild and farmed fish populations. Enteromyxosis caused by *Enteromyxum scophthalmi* (Palenzuela et al., 2002) is among the most severe parasitic diseases in mariculture, producing serious loses in turbot *Psetta maxima* (L.) (Branson et al., 1999; Quiroga et al., 2006). *E. scophthalmi* is highly specific for the digestive tract, mainly for the intestine, the target organ.

Infection by pathogens is generally initiated by the specific recognition of host epithelia surfaces. Receptors present in the mucin layer can act as binding sites in the subsequent adhesion, which is essential for invasion. The lectin/glycoconjugate interactions, characterized by their high specificity, are known to play a significant role in the adhesion of bacteria and parasites and in their interaction with the host. In their infection strategy, microorganisms often use sugar–binding proteins, such as lectins and adhesins to recognize and bind to host glycoconjugates (Imberty and Varrot, 2008). The blocking or inhibition of microbial lectins by suitable carbohydrates or their analogous is the aim of anti-adhesion therapy for the prevention and treatment of infectious diseases (Sharon, 2006). In addition, many carbohydrate residues present on the surface of parasites are specifically recognised by host lectins (Jacobson and Doyle, 1996; Nyame et al., 2004; Hammerschmidt et al. 2005). These carbohydrate structures of parasites can be used as prototypes for the chemical or combined chemo-enzymatic synthesis of new compounds for diagnosis and vaccine development, or as inhibitors specifically designed to target glycan byosinthesis (Mendoça-Previato 2002). Previous studies have demonstrated the binding of several plant lectins to carbohydrate residues present in *E. scophthalmi* stages (Redondo et al. 2008) and in the epithelial surface of

turbot intestine, and a role of lectin-carbohydrate interaction in the turbot-*E. scophthalmi* relationship has been suggested (Redondo and Alvarez-Pellitero 2009).

The unavailability of *in vitro* cultures of myxozoans, makes particularly difficult the studies on the interaction parasite/host. However, in the case of *E. scophthalmi*, the life cycle of the parasite can be experimentally maintained *in vivo* using effluent, cohabitation or oral infections (Redondo et al. 2002). In addition, turbot intestinal explants have been used *in vitro* to demonstrate the adhesion and penetration of this parasite into the intestinal epithelium (Redondo et al. 2004). In the present work, the involvement of the lectin/carbohydrate interaction in the invasion of the epithelium by *E. scophathalmi* was studied *in vitro* using explants of turbot intestine and pre-treatment of parasite stages with *Canavalia ensiformis* (Con A) and *Glycine max* (SBA), the two lectins showing the highest binding activity to *E. scophtalmi* (Redondo et al. 2008).

2. Materials and methods

Four trials using different methods of parasite treatment and incubation were performed (see below and Table I).

2.1. Intestine explants

Healthy turbot (*Psetta maxima*) were obtained from an *E. scophthalmi*-free farm. Fish were killed by overexposing to MS222 and bled from the caudal vein. After necropsy, portions of anterior or medium parts of intestine were collected, placed in sterile phosphate buffer saline (PBS) containing 2 x PSA antibiotic/antimycotic mixture (1x PSA = 100 $U.ml^{-1}$ penicillin, 100 $\mu g.ml^{-1}$ streptomycin, and 0.25 $\mu g.ml^{-1}$ amphotericin B), and cut in small pieces up to 10 x 6 mm. Small intestine pieces were placed (epithelial layer facing upwards) in each well of 24-well tissue culture plates

(one piece per well), containing 800 µl of PBS (trial 1) or Leibovitz's L-15 medium supplemented with 10% heat-inactivated foetal bovine serum (FBS) and 1 x PSA (trials 2, 3, 4). L-15 osmolarity was adjusted to 350 $mOsm.kg^{-1}$ by the addition of NaCl and pH to 7.2 with 20 mM HEPES.

All the experiments were carried out according to national (Royal Decrete RD1201/2005, for the protection of animals used in scientific experiments) and institutional regulations (CSIC, IATS Review Board) and the current European Union legislation on handling experimental animals

2.2. Source of parasites

Live *E. scophthtalmi* stages were obtained from turbot experimentally infected at the Instituto de Acuicultura Torre de la Sal (IATS) facilities. Infected fish were initially obtained from spontaneous infections occurring in turbot farms of Galicia, North West Spain (Redondo et al. 2004; Quiroga et al. 2006). This infection was experimentally transmitted via effluent water from tanks containing diseased fish; *per os*, by feeding infected intestinal tissue; and via cohabitation of infected and uninfected fish (Redondo et al. 2002). Since the experimental infection model was obtained, the life cycle has been maintained *in vivo* at the IATS facilities by several series of cohabitation of infected and uninfected fish or by oral infection.

2.3. Isolation and processing of E. scophthalmi stages

Infected fish were killed as above, necropsied and processed as explained previously (Redondo et al. 2002). Briefly, the intestinal fluid was collected from infected fish using a syringe and deposited in 15-ml centrifuge tubes containing PBS supplemented with a 2 x PSA. A drop of the intestinal liquid was observed as a fresh

smear at the microscope at 300x and the infection intensity was evaluated using the microscope at 300x magnification. Samples rich in parasite stages were centrifuged 10 min at 365 *g*. The pellet was processed in a different way depending on the trial (see below and Table I). In trials 1 and 2, the pellet was washed once in fresh PBS containing 2 x PSA. In trial 3, two further washes were done. In trial 4, a slight enzymatic treatment was applied after the third washing as follows: Stages were incubated in PBS containing 0,370 mg/ml EDTA (ethylenediaminetetraacetic acid) and 0,145 mg/ml DTT (dithiothreitol) during 1 h at 18ºC under shaking, and washed in washing medium (HBSS pH 7.2, 5% FBS, 1 x PSA, 0.1mg/ml DNase I). After filtration using a 40 µm cell stainer, the filtrate containing the parasites was resuspended in L-15 medium. In all cases, the stages in the final pellets were counted and their viability estimated using eosin dye-exclusion methods. The parasites present in the pellets belonged to stage 2 (a primary cell containing one or several secondary cells) and stage 3 (a primary cell containing one or several secondary cells, which in turn harboured one or more tertiary cells), according to Redondo et al. (2004). All the procedure was carried out using sterile material and aseptic techniques.

2.4. Parasite culture with intestine explants

In the four experiments, the isolated parasites were divided into three parts. Two of them were incubated with 50 µg/l Con A or SBA, recognising Man α-1>D-Glc α-1>GlcNAc α-1 and α,ß GalNAc>α,ßGal residues, respectively, during 30', and the third one served as control, non-incubated with the lectin. Details on the parasite obtaining, temperature and incubation conditions can be found in Table I. The lectin-incubated and control parasite suspensions ($2.5x10^5$-$1x10^6$ stages/ml) were added to the wells containing the intestinal explants. Control wells contained intestine pieces with no

parasites added. After incubation at 15-18ºC, intestine portions were recovered at 2-3 h post-exposure (p.e.), fixed in 10 % neutral buffered formalin and embedded in Technovit-7100 resin (Kulzer, Heraeus, Germany). Sections (2 µm) were stained with toluidine blue. The presence of parasite stages was evaluated in the histological sections at light microscope by counting the number of parasites that appeared attached or within the epithelia. For such purpose, serial sections of the epithelial tissue were obtained and sets of 4 sections per slide were mounted on successive slides. In order to assure the examination of different levels of the tissue and to avoid repetition of stages, the four sections of impair slides were examined. In each section, 32 observational fields of epithelium (460 x 460 µm surface) were seen at 300x. Three replicate series of counts were done per condition. In each replicate, 4 slides (16 sections, 108 mm^2 of epithelial surface) were examined, and the parasites attached or within the epithelium were counted.

2.5. Statistical analysis

Differences between the three conditions in each trial and between trials were analysed by One-way analysis of variance (ANOVA). When the test of normality or equal variance failed, a Kruskal-Wallis one-way ANOVA on Ranks followed by Tukey test or Holman-Sidak method was applied instead. All statistical analyses were performed using Sigma Stat software (SPSS Inc., Chicago, IL, USA), and the minimum significance level was set at $P<0.05$.

3. Results

The number of *E. scophthalmi* stages attached to or penetrated in the turbot intestinal explants was higher for control parasites than for stages pre-incubated with

both Con A and SBA, with a more pronounced effect of SBA (Fig. 1). Differences were statistically significant for SBA-treated stages with respect to untreated stages in all trials except in trial 4. However, the number of Con A treated stages was significantly lower than that of untreated stages only in trial 3.

Few stages were observed within the epithelium in any treatment, but their number was again lower for Con A treated parasites whereas none stage was seen in this location after SBA treatment. Attached (Figs. 2A-B) or internalised (Fig. 2C) stages were mainly stage 3 parasites (Figs. 2A, C), though some stages with the appearance of secondary cells released from a mother stage were seen adhered to epithelium (Fig. 2D). Lectin treated stages appeared sometimes in close contact, suggesting an agglutinating effect of the lectin (Fig. 2E). As the number of attached or penetrated stages in control explants was low in trials 1 and 2, some conditions were changed in subsequent trials to improve the invasion. The number of attaching or invading stages was the highest in trial 3, when using washed stages at 15 ºC, though the difference was statistically significant only with respect to trial 1. The enzymatic treatment to separate the stages from the mucus (trial 4) did not improve the invasion and penetration. In spite of the observed differences between trials, the effect of the pre-incubation of parasite stages with lectins was inhibitory in all cases.

4. Discussion

The incubation of *E. scophthalmi* stages with Con A and SBA influenced their ability to invade the epithelium of turbot intestinal explants *in vitro*. The inhibitory effect was higher for SBA than for Con A, which points to the involvement of GalNAc-Gal and also of Man/Glc residues in the *E. scophthalmi*/intestinal epithelium interaction. Such effect was confirmed in several experiments using parasite material obtained from

different individual fish and performed in different conditions. The use of washed stages at 15 ºC proved to be the most efficient condition for these *in vitro* studies.

The obtained results indicate that such residues could be recognised by putative lectins present in the fish intestinal epithelium. Among animal lectins recognising Man residues, the best characterized is the mannose-binding lectin (MBL), a C-type lectin that plays an essential role as initiator of the primary immune response and participates in inflammation (Petersen et al. 2001; Turner 2003; Klein 2005; Arnold et al. 2006). MBL or its homologous exist in different organs of teleosts, including the intestine (Vitved et al. 2000; Nikolakopoulou and Zarkadis 2006). The pufflectin, another MBL, is present in the mucosal tissues of skin and digestive tract of pufferfish and can bind specifically to the monogenean *Heterobothrium okamotoi* (Tsutsui et al. 2003). Other animal lectins recognise Gal residues, such as galectins and intelectins. There are several mammalian galectines, some of them expressed in the intestine (Rabinovich 1999). Among piscine galectins, the congerin, located at skin and mucosal tissues (Nakamura et al. 2001) can play immune functions in the Japanese conger eel (*Conger myriaster*) intestinal lumen (Nakamura et al. 2007). Intelectins are Ca2+-dependent Gal binding lectins that are expressed in Paneth and goblet cells of mammalian small intestine (reviewed in Nair et al. 2006; Wrackmeyer et al. 2006). Several intelectins have been characterized in the fish gut and some of them can interact with bacteria (reviewed in Alvarez-Pellitero 2009).

Several transmembrane or soluble lectin domains in the host can interact with Man, fucose or Gal structures in different parasites and can thus play a crucial role in the host/parasite interaction and invasion, and also in the immune evasion (Cambi and Figdor 2005). Among lectins recognising D-Man and D-Glc residues, MBL is known to be involved in the interaction and response to some parasites (Klabunde et al. 2000;

Ambrosio and Messias-Reason 2005; Hokke and Yazdanbakhsh 2005; Gruden-Movsesijan and Milosavljevic 2006). In addition, *in vitro* experiments have demonstrated a reduction of the invasion of macrophages by *Leishmania mexicana* (Bray 1983) and of the excised skin of channel catfish by the ciliate *Ichthyophtyrius multifiliis* (Xu et al. 2001) by lectins recognising such residues.

Among lectins recognising Gal residues, intelectins have been also proposed to have a role in the response to parasite infections. Intelectin-1 and intelectin-2 are up-regulated in mice infected with *Trichuris muris* and *Trichinella spiralis*, respectively (Pemberton et al. 2004; reviewed in Artis and Grencis 2008). Lectins binding to O-linked glycoconjugates containing Gal or GalNAc residues mediate inhibition of sporozoite infectivity of *Cryptosporidium parvum in vitro* (Gut and Nelson 1999). Other studies have confirmed the role of lectin carbohydrate interactions involving a Gal/GalNAc-specific lectin in *C. parvum* attachment to epithelial cells (Chen and LaRusso 2000) and the role of GalNAc residues in blocking the attachment to host cells (Cevallos et al. 2000). In a similar manner, the penetration of sporozoites of *Eimeria tenella* was suppressed when pretreated with peanut lectin that specifically recognizes D-Gal residues (Baba et al. 1996).

The currently obtained results demonstrated also the adequacy of turbot intestinal explants as an *in vitro* model to study the interaction with *E. scophthalmi*. The inhibitory effect of the studied lectins on the parasite adhesion and penetration points to the interest of further studies to confirm the presence of putative lectins recognising *E. scophthalmi* structures in turbot intestine. The potential use of lectins in invasion-inhibiting treatments, as suggested for *C. parvum* (Gut and Nelson 1999; Cevallos et al. 2000) and *Giardia lamblia* (Ortega-Barria et al. 1994; Grant et al. 2001) infections deserves further investigations.

Acknowledgements

This work was supported by the Spanish Ministry of Science and Education through the research projects AGL2006-13158-C01 and AGL2009-13282-C02-01. María J. Redondo is recipient of a CSIC I3P contract funded by the European Social Fund. We are thankful to Mr. J. Monfort and L. Rodríguez for light microscope processing.

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Figure legends

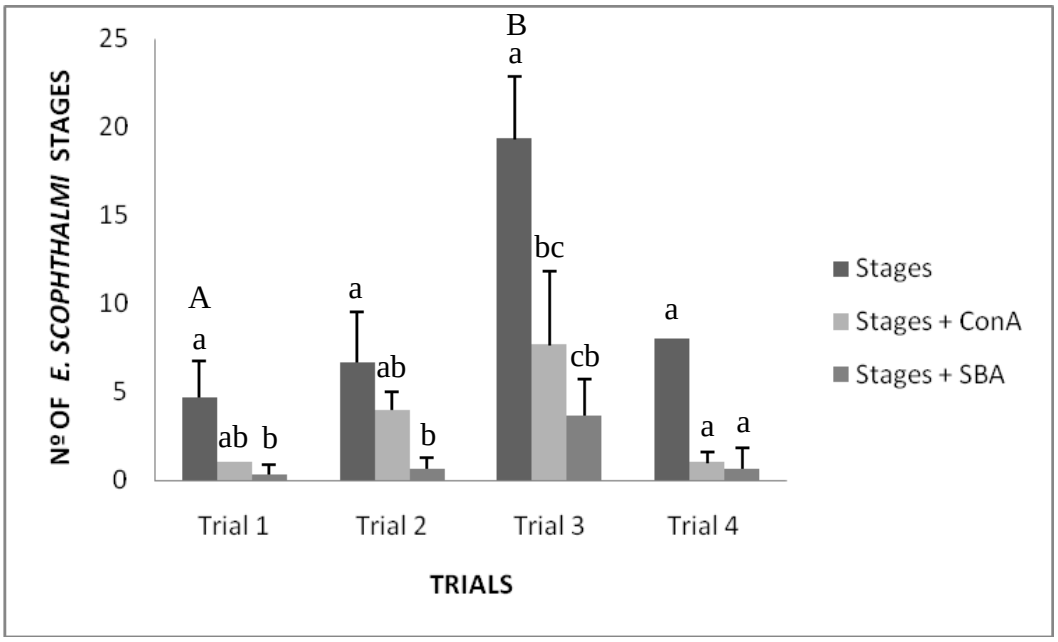
Fig. 1. Number of *E. scophthalmi* stages (mean ± SE) attached or penetrated in the turbot intestinal explants *in vitro* in trials 1 to 4. Different low-case letters indicate statistically significant differences ($P \leq 0.05$) between parasite treatments within each trial. Capital letters indicate statistically significant differences between trials. Stages: control. Stages + Con A, Stages + SBA: pre- incubated with Con A or SBA, respectively.

Fig. 2. Histological sections of intestinal explants of turbot after exposure to *E. scophthalmi* stages non-incubated (control) or pre-incubated with the plant lectins ConA or SBA. **A.** Trial 2, control. The parasite adhered to the epithelial surface (stage 3) seems to initiate the penetration in the epithelium. **B.** SBA. Trial 3. Stage 3 with the membrane of the primary cell adhered to the epithelium. **C-E.** Con A. **C.** Trial 1. Stage 3 within the epithelium. Two secondary cells (containing tertiary cells) can be seen in a primary cell. **D.** Trial 3. Two stages 3 (arrows) are near the mucus layer. In the upper part of the image, three secondary cells apparently released from the primary cell appear attached to the brush border (arrowhead). **E.** Trial 2. Three stages are closed together, apparently agglutinated. Staining: Toluidine blue. Bars: Figs. A, D-E: 20 µm; Figs. B-C: 10 µm.

Table I. Experimental conditions in the four trials of *in vitro* infection of *E. scophthalmi* in turbot intestine explants

Conditions	Trial 1	Trial 2	Trial 3	Trial 4
Parasite stages processing	Washed once in PBS	Washed once in PBS	Washed trice in PBS	Washed trice in PBS + enzymatic treatment
Incubation medium	PBS	L-15	L-15	L-15
Stages/ml	10^6	10^6	$2.5x10^5$	$2.5x10^5$
Temperature	20ºC	20ºC	15ºC	15ºC
Incubation time	2h	2h	3h	3h

Figure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TRIALS
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Figure

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