ULTRASTRUCTURAL LOCALISATION OF CARBOHYDRATES IN FOUR MYXOSPOREAN PARASITES

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Summary :

The existence and localisation of carbohydrates in four myxosporean parasites was investigated at transmission electron microscope, using lectin histochemistry techniques. The Myxosporea studied showed different lectin binding patterns. N-acetyl-glucosamine or its polymers were predominant in the valves of *Leptotheca* sp. and were also present in *Sphaerospora dicentrarchi* and *Polysporoplasma sparisi* ones. Mannose and/or glucose terminals were mainly detected in *S. dicentrarchi* and *P. sparisi* valves. Polar capsule walls were intensively recognised by Con-A in *S. dicentrarchi* and with medium intensity in *P. sparisi* and *Leptotheca* sp. The polar filament was scarcely labelled except in *Leptotheca* sp. with BS-I. The sporoplasms of the studied parasites were stained with all the lectins tested with each myxosporea, except those of *Leptotheca* sp. with BS-I. Some structures of the developmental stages of *Zschokkella mugilis* and *Leptotheca* sp. were stained with BS-I. The possible role of these carbohydrate terminals found in the studied Myxosporea is discussed.

KEY WORDS : lectins, ultrastructure, histochemistry, Myxozoa, Teleostei.

Résumé : LOCALISATION ULTRASTRUCTURALE DE GLUCIDES CHEZ QUATRE MYXOSPORIDIÉS PARASITES

Une étude ultrastructurale précise la présence et la localisation de fractions glucidiques chez quatre myxosporidies par histochimie avec lectines. Les myxosporidies étudiées montrent différents patrons de reconnaissance avec les lectines. On observe une prédominance de N-acétylglucosamine ou de ses polymères dans les valves de *Leptotheca* sp. et sa simple présence dans les valves de *Sphaerospora dicentrarchi* et *Polysporoplasma sparisi*. Les terminaisons mannose et/ou glucose sont surtout trouvées dans les valves de *S. dicentrarchi* et *P. sparisi*. Les parois des capsules polaires sont intensivement reconnues par la Con-A dans *S. dicentrarchi* et avec une moindre intensité dans *P. sparisi* et *Leptotheca* sp. Les filaments polaires montrent un léger signal, exception faite de ceux de *Leptotheca* sp. intensivement marqués avec BS-I. Les sporoplasmes des parasites étudiés ont été marqués par toutes les lectines trouvées dans chaque parasite, excepté ceux de *Leptotheca* sp. avec BS-I. Cette lectine marque quelques structures des stades de développement de *Z. schokkella mugilis* et *Leptotheca* sp. On discute le rôle des fractions glucidiques trouvées dans les myxosporidies étudiées, et on suggère la nécessité de nouvelles études pour éclaircir leur implication dans les relations hôte-parasite.

MOTS CLÉS : lectines, ultrastructure, histochimie, Myxozoa, Teleostei.

INTRODUCTION

The interest in the culture of commercial fish has emphasised the importance of pathological processes. Parasitic diseases are responsible for important economical losses in aquaculture (Bauer *et al.*, 1981). Among these, some Myxosporea have a significant impact on freshwater (El-Matbouli *et al.*, 1992) and marine (Álvarez-Pellitero & Sitjà-Bobadilla, 1993; Company *et al.*, 1999) fish. Nevertheless, many questions about their biology and physiology remain unanswered, and the study of their glycoconjugates is very limited. Although several authors have studied the myxosporean carbohydrate topography using lectin

immunohistochemical methods at light microscope (Hedrick *et al.*, 1992; Lukes *et al.*, 1993; Marín de Mateo *et al.*, 1996, 1997; Muñoz *et al.*, 1999), the ultrastructural distribution of these components in Myxosporea has not yet been investigated.

Considering the likely importance of polysaccharides of an invading pathogen in the defence reaction of the host, in the current work we have examined the ultrastructural saccharide patterns of several myxosporean parasites, using lectin labelling techniques.

MATERIALS AND METHODS

TISSUE SAMPLES

Periodic surveys of sea bass (*Dicentrarchus labrax* L.), gilthead sea bream (*Sparus aurata* L.), grey mullet (*Mugil cephalus* L.) and common dentex (*Dentex dentex* L.) were conducted for myxosporean

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parasite examination. They included fish of different stocks and ages from the Mediterranean area. Fish were overexposed to the anaesthetic MS-222 (Sigma Chemical Co., St. Louis, MO, USA), necropsied, and their organs excised for fresh and histological diagnosis. Parasitised organs were selected for ultrastructural studies.

ULTRASTRUCTURAL LECTIN HISTOCHEMISTRY

Sea bass intestines infected by *Sphaerospora dicentrarchi*, gilthead sea bream trunk kidneys harbouring *Polysporoplasma sparis*, grey mullet gall bladders infected by *Zschokkella mugilis*, and common dentex trunk kidney with *Leptotheca* sp. were fixed in 1 % (v/v) glutaraldehyde and 4 % (w/v) paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2, 4°C), post-fixed in 0.1 % (w/v) cacodylic OsO₄, dehydrated through a graded ethanol series, and embedded in Lowicryl K4M (Polysciences Ltd.). Ultrathin sections were collected on 200 mesh gold grids coated with Formvar. Grids were incubated with blocking buffer (1 % (v/v) bovine serum albumin (Sigma) in 10 mM Hepes, pH 7.5) at 20°C for 15 min, and with biotinylated lectins at 20°C overnight. The concentrations of the lectins used in this study, their acronyms and major sugar specificities are listed in Table I. All the lectins were used following the manufacturer's instructions. *Griffonia simplicifolia* lectin (BS-I) (Vector Lab., Burlingame, CA, USA) was diluted in lectin buffer (10 mM Hepes, 0.15 M NaCl, 0.1 mM CaCl₂, pH 7.5), *Canavalia ensiformis* lectin (Con-A) (Vector) in lectin buffer plus 0.01 mM MnCl₂, *Sambucus nigra* lectin (SNA) (Vector) in lectin buffer plus 0.008 % sodium azide, and *Triticum vulgaris* lectin (WGA) (Sigma) in 0.05 M Tris, 0.15 M NaCl, 1 mM MnCl₂, 1 mM MgCl₂, 0.1 mM CaCl₂, 0.5 mg/ml polyethylene glycol, pH 7.5. In all cases, the lectin concentration used was 5 µg/ml. After rinsing in 10 mM Hepes, grids were incubated with streptavidin conjugated with 15 nm colloidal gold particles (Biocell) in 10 mM Hepes (1:25) at 20°C for 1 h. Negative controls were obtained using 10 mM Hepes instead of each lectin in the first incubation. Grids were washed with 10 mM Hepes and bidistilled water, and stained with uranyl acetate and lead citrate. They were examined in a Hitachi H-800 MET operating at 80 kV. An equal amount of each lectin and its corresponding blocking sugar (0.2 M) were incubated overnight at 20°C, before application of the solutions to the grids, as a control for binding specificity. The blocking sugars used were lactose (Sigma) for BS-I, α -N-acetyl-D-glucosamine (Sigma) for WGA, methyl- α -D-mannopyranoside (Sigma) for Con-A, and N-acetylneuraminic acid (Sigma) for SNA. Each parasite was assayed with the lectins which gave the clearest positive results at light microscope (Muñoz *et al.*, 1999), as listed in Table I.

Structure labelled	Capsulogenic cells	Polar capsule walls	Polar filaments	Sporoplasms	Valves	Developmental stages
Parasites	<i>Leptotheca</i> sp.	++	-	++	-	Nf
	<i>P. sparis</i>	+	+	+	+	Nf
	<i>S. dicentrarchi</i>	-	+	+	+	Nf
	<i>Z. mugilis</i>	Nf	Nf	Nf	Nf	Nf
	<i>Leptotheca</i> sp.	++	++	+	+	++
	<i>P. sparis</i>	++	+	+	+	Nf
	<i>S. dicentrarchi</i>	+	+	++	+	Nf
	<i>Z. mugilis</i>	Nf	Nf	Nf	Nf	++
	<i>Leptotheca</i> sp.	+	+	+	+	Nf
	<i>P. sparis</i>	++	+	+	+	Nf
	<i>S. dicentrarchi</i>	+	+	++	+	Nf
	<i>Z. mugilis</i>	Nf	Nf	Nf	Nf	++
Lectins*	SNA	-	-	-	-	-
	BS-I	+	+	+	+	+
	WGA	+	+	+	+	+
	Con-A	++	++	++	++	++

* SNA = *Sambucus nigra*, specific for Neu5Ac α -2,6 Gal > GalNAc; BS-I = *Griffonia simplicifolia*, specific for D-Gal > D-GalNAc; WGA = *Triticum vulgaris*, specific for (GlcNAc β -1,4)_n > NeuNAc; Con-A = *Canavalia ensiformis*, specific for Man α -1 > Glc α -1 > GlcNAc α -1.

Abbreviations: GalNAc = N-acetyl-galactosamine; Gal = galactose; GlcNAc = N-acetylglucosamine; NeuNAc = neuraminic acid; Man = mannose; Glc = D-Glucose.

Table I. - Summary of lectin binding patterns found in myxosporean parasites. Intensity of staining: +++ = intense; ++ = moderate; + = weak; - = none; Nf = stages not found in the sections. Blanks correspond to not tested lectins due to previously found negative at LM.

RESULTS

The ultrastructural localisation of polysaccharides in myxosporean parasites by different lectins is summarised in Table I.

Con-A stained moderately the capsulogenic cells and the polar capsule wall, and weakly the sporoplasms of *Leptotheca* sp. Some labelling was observed in the cytoplasm, the nucleus and the mitochondria of capsulogenic cells in immature spores with this lectin (Figs. 1, 2). WGA labelled the valves of this parasite and very weakly their sporoplasms. BSI bound strongly to the polar filaments and weakly to the polar capsule walls and the valves of *Leptotheca* sp. (Fig. 3). In developmental stages, moderate labelling was seen in the cytoplasm of primary and secondary cells.

Con-A bound moderately to the polar capsule wall of *Polysporoplasma sparis* spores and to their valves and sporoplasms. WGA labelled moderately the polar capsule wall, and weakly the capsulogenic cells, the polar filament, the sporoplasms and the valves of this parasite (Fig. 4). SNA stained moderately the sporoplasms (Fig. 5), the polar capsule wall and the capsulogenic cells of *P. sparis*, and weakly the polar filaments. Some gold particles were also observed in mitochondria of sporoplasms and capsulogenic cells with these three lectins, mainly with SNA.

Sphaerospora dicentrarchi lectin histochemistry with Con-A (Fig. 6) resulted in a strong staining of sporoplasms and polar capsules (mainly the walls), and a moderate labelling of valves. WGA bound moderately to the sporoplasms of *S. dicentrarchi* and weakly to capsulogenic cells, valves and the polar capsule wall (Fig. 7). SNA stained weakly the sporoplasms, the polar capsule walls and the polar filaments of this parasite.

BSI stained some structures of *Zschokkella mugilis* developmental stages (Figs. 8, 9). The most intense staining was located in the nucleus. Some scarce gold particles were scattered in the cytoplasm of the primary, secondary and tertiary cells, and also in some of their mitochondria.

DISCUSSION

Due to the multifaceted properties of lectins, they have been used as probes to investigate cell surface structures and functions (Slifkin & Doyle, 1990). Using ultrastructural studies, information can be obtained not only about the presence of specific carbohydrates but also on their location. The Myxosporea studied showed different ultrastructural lectin binding patterns. Previous observations at light microscope (LM) on the same parasites (Muñoz *et al.*,

1999) demonstrated that valves and polar capsules are the main stained structures with the different lectins. This general staining pattern was in part confirmed by the present ultrastructural observations. The exact binding location was accurately determined and some differences were seen with respect to the LM observations, mainly regarding the staining intensity.

Parasitic surfaces are involved in many host-parasite interactions, and are made up of oligosaccharide side chains of glycoproteins and glycolipids (Peters, 1988). In our study, spore valves were stained mainly with two lectins, WGA and Con-A. WGA stained them in all the parasites assayed. This lectin recognises mainly N-acetyl-glucosamine and, to less extent, sialic acid. In *Sphaerospora dicentrarchi*, the absence of binding to the valves with SNA (already observed at LM, Muñoz *et al.*, 1999) was confirmed in this ultrastructural study. Thus, the presence of N-acetyl-glucosamine or its polymers was demonstrated. Con-A bound to the valves of two parasites, *Sphaerospora dicentrarchi* and *Polysporoplasma sparis*, though moderately. Therefore, some mannose and/or glucose terminals must also be present. Thus, this ultrastructural study demonstrated the predominance of N-acetyl-glucosamine or its polymers in the valves of *Leptotheca* sp. and its presence in *P. sparis* and *S. dicentrarchi*, whereas mannose and/or glucose terminals were mainly present in *S. dicentrarchi* and *P. sparis* valves. It is probable that N-acetyl-glucosamine or its polymers have a protective and structural role in these spores. Chitin, the polymer of N-acetyl-glucosamine, is a structural component of the wall of many fungi and it can show a different distribution pattern in the wall parts, as in the deuteromycete *Chalara elegans* (Dumas-Gaudot, 1992) or the hyphomycete *Nomuraea rileyi* (Pendland & Boucias, 1992).

Polar capsules constituted another frequently stained structure with the different lectins used in the present study. The wall of the polar capsules was intensively recognised by Con-A in *S. dicentrarchi*, and with medium intensity in *P. sparis* and *Leptotheca* sp., whose capsulogenic cells (mainly in immature spores) were also stained. Polar filaments were scarcely recognised by the different lectins, and the most intense staining was obtained with BSI in *Leptotheca* sp. Thus, galactose and/or N-acetyl-galactosamine seem to be present in this structure. In addition, only SNA detected slightly the polar filament of *P. sparis* and *S. dicentrarchi*.

An outstanding result of this ultrastructural study is the staining of the sporoplasms of most parasites with all the lectins tested with each myxosporea, except those of *Leptotheca* sp. with BS-I. However, differences were observed in their staining pattern. The strongest binding was obtained with Con-A in *S. dicentrarchi*, and

Figs. 1 to 4. – Transmission electron microscopy images of myxosporean parasites in tissue sections stained with lectins. Figs. 1 to 3. *Leptotheca* sp. from common dentex trunk kidney. Figs. 1, 2. Sections of immature spores stained with Con-A. **Fig. 1.** – Note the labelling in mitochondria (arrowheads), in the cytoplasm (*), in the capsular primordium (arrow) and in the external tube. **Fig. 2.** – Note the scarce labelling in the nucleus of a capsulogenic cell (arrows) and a group of gold particles associated to rough endoplasmic reticulum (arrowhead). **Fig. 3.** – Section of a mature spore stained with BS-I. A distinct labelling in the polar filament is observed (arrowhead). **Fig. 4.** – *Polysporoplasma sparis* in gilthead sea bream trunk kidney stained with Con-A. Note the staining of the polar capsule wall (arrowheads) and valves. Bars = 0.2 μ m.

Figs. 5 to 7. – Transmission electron microscopy images of myxosporean parasites in tissue sections stained with lectins. **Fig. 5.** – *Polysporoplasma sparis* in gilthead sea bream trunk kidney stained with SNA. Note the scattered labelling of the sporoplasms (S) and the very scarce staining in their mitochondria (arrowhead). **Figs. 6, 7.** *Spbaerospora dicentrarchi* in sea bass intestine. **Fig. 6.** – Staining with Con-A. Strong labelling in polar capsules and moderate staining in valves. **Fig. 7.** – Staining with WGA. Moderate staining in sporoplasms and weak labelling of polar capsule walls (arrow) and valves (arrowheads). Bars = 0.2 μ m.

Figs. 8, 9. – Transmission electron microscopy images of *Zschokkella mugilis* in a mullet gall bladder section stained with BS-I. Fig. 8. – Primary cell (P) attached to the epithelium of the host (H), containing secondary (S) and tertiary (T) cells. Bar = 1 μ m. Fig. 9. – Detail of these S and T cells showing the scarce labelling in the cytoplasm and mitochondria (arrows) and some staining in the nucleus (arrowheads). Bar = 0.1 μ m.

a medium intensity of staining was detected with Con-A and SNA in *P. sparis*, and with WGA in *S. dicentrarchi*.

Only in the case of *Zschokkella mugilis* and *Leptotheca* sp. were some developmental stages observed in the sections and some of their structures were stained with BS-I. D-galactose or N-acetyl D-galactosamine residues have also been detected in the developmental stages of other myxosporean parasites such as PKX (the causative agent of proliferative kidney disease) (Marín de Mateo *et al.*, 1997).

The ultrastructural binding pattern of a polyclonal antiserum raised against *S. dicentrarchi* (Muñoz *et al.*, 1998) was, in part, coincident with that obtained with Con-A. As the carbohydrate components of glycoproteins can be antigenic determinants (Rafferty & Mulcahy, 1988; Roth *et al.*, 1997), it seems possible that some of the epitopes recognised by this polyclonal antiserum could be the carbohydrates detected in this study.

The most important residues, which probably play a role in the host-parasite interaction, include mannose/glucose, sialic acid and galactose, as it has been demonstrated in different pathogens. Mannose and/or glucose have been detected by Con-A binding in other fish parasites, as *Cryptobia* spp. (Feng & Woo, 1998). Some mammalian parasites, as *Entamoeba histolytica*, *Acanthamoeba* sp., *Trichomonas vaginalis*, *Pneumocystis carinii* or *Leishmania* spp. (see Slifkin & Doyle, 1990) also showed Con-A-mannose interactions which have been associated to virulence or infectivity in some cases. Moreover, the role of macrophage man-

nose receptors in innate defence against a variety of microorganisms is well known (Drickamer & Taylor, 1993; Holmskov *et al.*, 1994). Sialic acids are important regulators of cellular and molecular interactions, and they can mask recognition sites or serve as recognition determinants. However, little is known about the role of sialic acids in the biology of parasites. Galactose plays a role in fungi-insect interactions (Penland & Boucias, 1993) or can act as a storage product (Dumas-Gaudot *et al.*, 1992).

The terminals demonstrated in our Myxosporea might also play a role in the host-parasite relationship. Those found in the spore surface or in polar filaments could be involved in the invasion and dispersion within the hosts, including a putative invertebrate intermediate host, if the complex life cycle demonstrated for some fresh water myxosporean parasites (Wolf & Markiw, 1984; Bartholomew *et al.*, 1997; Lin *et al.*, 1999) takes also place in these marine myxosporoses. Different membrane and humoral lectins have been demonstrated in invertebrates (Vasta & Ahmed, 1996), and some of them are involved in host-pathogen interactions (Penland & Boucias, 1992; Welburn & Maudlin, 1999). Spores can also interact with the defence system of the fish host, especially in the case of inflammatory reactions. The developmental stages in the fish could also be involved in the host-parasite interaction, both in the dispersion of proliferative stages or in possible fish-to-fish infection. Therefore, further research is needed to clearly ascertain the role of the carbohydrate terminals in the myxosporean parasites and in the host-parasite relationship.

ACKNOWLEDGEMENTS

This work was supported by research grants from the Spanish Ministerio de Educación y Cultura No. AGF95-0058 and MAR98/1000. Pilar Muñoz received a grant from the Spanish Ministerio de Educación y Cultura. We are thankful to technicians from the Electron Microscopy Service of the University of Barcelona and to the Instituto Español Oceanográfico at Mazarrón (Murcia) for providing some of the fish.

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Reçu le 10 février 2000

Accepté le 31 mai 2000