Histol Histopathol (1998) 13: 37-45

Histology and Histopathology

Histochemical study of lymphocystis disease in skin of gilthead seabream, *Sparus aurata* L.

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Summary. A battery of horseradish peroxidase-conjugated lectins (Con A, WGA and DBA), as well as conventional histochemical techniques (PAS, saponification, Alcian Blue pH 0.1, 1, 2.5, chlorhydric hydrolisis, sialidase, Bromophenol blue, Tioglycollate reduction and Ferric-ferricyanide-FeIII) were used to study the content and distribution of carbohydrates, proteins and glycoconjugate sugar residues on the skin and on the lymphocystis-infected cells of gilthead seabream, *Sparus aurata*. Variable amounts of glycoproteins containing sialic acid, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, mannose and/or glucose residues were observed in the cuticle and mucous cells of the corporal skin, tails and fins. Germinative and epithelial cells of the epidermis contained glycogen, proteins, carboxylated groups, as well as glycoproteins with mannose and/or glucose and N-acetyl-D-galactosamine residues. Hyaline capsule of the mature lymphocystis-infected cells was strongly stained with PAS, Alcian Blue (pH 0.5 and 2.5) and weakly positive with Alcian Blue (pH 1). Con A reacted with the granular cytoplasm, specially around hyaline capsule, and with the basophilic intracytoplasmic inclusions developed in mature lymphocystis-infected cells of *Sparus aurata* skin. These sugar residues (mannose and/or glucose), as well as N-acetyl-D-glucosamine and/or sialic acid and N-acetyl-D-galactosamine were not detected in the hyaline capsule of the lymphocystis disease.

Key words: Skin, Lymphocystis disease, Glycoproteins, Carbohydrates, Proteins, Histochemistry, *Sparus aurata*

Introduction

The skin is the primary barrier against the environment, allowing normal internal physiological function, so its condition is important in many disease processes. The skin mucus of fishes may be important in natural defense against parasites and pathogenic microorganism (Fletcher, 1978) besides having a possible osmoregulatory and lubricant function (Van Oosten, 1957; Rosen and Cornford, 1971). The defense may be mechanical, due to continuous production of mucus (Pickering, 1974). However, the presence of lysozymes, complement components of the IgM type, suggests that the skin mucus takes and active part in the immunity system (Hjelmeland et al., 1983).

The layers of teleosts skin comprises cuticle, epidermis, basement membrane, dermis and hypodermis. The external layer, the cuticle, was first described in detail by Whitear (1970) as a mainly mucopolysaccharide layer approximately 1 μm thick. It is normally secreted largely by epithelial surface cells rather than by goblet cells and is a complex of cell protoplasm, sloughed cells and any goblet cell mucus that has been secreted onto the surface. The cuticular layer contains specific immunoglobulins, lysozyme and free fatty acids, all of which are believed to have antipathogen activity. The epidermis, which originates from the ectoderm, can be further divided into two layers. The outer layer is the epithelial cell layer that belongs to the stratified type of epithelium. The basement of this layer or germinal layer, is composed of undifferentiated cells (Roberts, 1978; Hibiya, 1982).

Goblet cells produce the mucus which covers the body and these mucous cells are usually present in the epidermis of fishes. In *Anguilla japonica*, *Anguilla anguilla* and *Misgurnus anguillicaudatus*, a large cell with no clear function, clavate cell, can also be recognized. Clavate cells contain some proteins and show a weak PAS-positive reaction (Roberts, 1978; Yamada and Yokote, 1985; Sarasquete et al., 1989). Mucous cells are differentiated from basal cell, and gradually approach the surface region, at the same time increasing in size and elaborating secretions, mainly glycoproteins (Roberts, 1978). In different species, mucous cells contain sialic acid (Askawa, 1970; Harris et al., 1973; Hibiya, 1982; Sarasquete et al., 1989; Illana, 1993), as well as sulphated glycoproteins (Fletcher and Grant, 1968; Sarasquete et al., 1989). Sialic acid has

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been used to estimate the degree of skin mucification in fishes (Lemoine and Olivereau, 1971; Pickering, 1974) and mucus secretion can be an indicator of pathological or inflamatory processes induced by the environment (Arillo et al., 1979). Sialisation and sulphation of the glycoproteins may be important for increasing the resistance of mucus to bacterial degradation (Rhodes et al., 1985).

In higher vertebrates, the cell surface glycoproteins have been shown to play important roles in pinocytosis, differentiation, intercellular recognition, as receptor for hormones and virus and as mediators of immunological specificity (Olden et al., 1982). Lectins are glycoproteins of non immune origin with the ability to recognize specific saccharides, such as N-acetylneuraminic acid or sialic acid (NANA), mannose (Man), glucose (Glc), galactose (Gal), fucose (Fuc), N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) residues, etc. In the skin of different teleosts, Illana (1993) showed the presence of glycoproteins containing different sugar residues. The mucous cells of *Anguilla anguilla* and *Halobatrachus didactylus* are specially rich in sialic acid; the absence of ß and α-galactose (Gal), N-acetylgalactosamine (GalNAc) and α-L- fucose (Fuc) sugar residues were pointed out in epithelial germinative and/or goblet cells of the skin of these species.

On the other hand, lymphocystis is a chronic viral disease reported in captive and wild marine and fresh water fish from different geographic areas (Amin, 1979). The first sign of infection is rounding of hypertrophied fibroblasts which grow rapidly and develop dark inclusions within the cytoplasm (Roberts, 1978). In *Sparus aurata*, Paperna et al. (1982) and González de Canales et al. (1996) showed, respectively, the viral etiology of this pathology and some histochemical characteristics of lymphocystis disease. The presence of sulphated and carboxylated glycoproteins in the hyaline capsule, and the protein coat of the virus particles containing SH and S-S groups, has been reported in different species (Walker and Weissenberger, 1965; Howse and Christmas, 1970; González de Canales et al., 1996). The interest of the glycoproteins as viral receptors was pointed out by Olden et al. (1982). According to Rogers et al. (1986), some virus utilize sialic acid as the primary receptor determinant for attachment to cell receptors.

In this paper, we report the histochemical characteristics (glycoproteins, carbohydrates and proteins) in the skin, as well as the presence of glycoproteins containing different sugar residues in lymphocystis-infected cells (cytoplasm, inclusions, hyaline capsule) of seabream, *Sparus aurata* from the south-atlantic coast of Spain.

Materials and methods

Juvenile specimens of the gilthead seabream, *Sparus aurata* L with and without external lymphocystis disease symptoms, were supplied by mariculture facilities of the south atlantic coast of Spain. Samples of corporal skin, tail and/or fins were fixed in Bouin solution and embedded in paraffin. Sections of 5-7 μm thick were stained with Haematoxylin-eosin and Haematoxylin-V.O.F (Sarasquete et al., 1993, 1995). Samples for scanning electron microscopy were fixed in 4% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2, dehydrated through ethanol series, critical point dried with liquid CO_2, coated with gold and viewed in a Hitachi S-570 scanning electron microscope. Cytochemical tests for carbohydrates (PAS, diastase-PAS; Alcian Blue pH 0.5, 1 and 2.5; neuraminidase-type V from *Clostridium perfringens*, Sigma- or chlrorydric hydrolisis; acetylation and saponification), glycoproteins (lectins) and proteins (Bromophenol blue, Ferric-Ferricyanide-FeIII and reduction with Tioglycollate), previously standarized in other tissues of fishes (Sarasquete et al., 1996) and described in Pearse (1985) and Bancroft et al. (1990) were performed.

For lectins, sections were treated with 0.3% hydrogen peroxide for 10 minutes (to inhibit the endogenous peroxidase) in Tris buffered saline (TBS) at pH 7.2. The sections were incubated for 30 minutes at room temperature in horseradish peroxidase-conjugated lectins (HPR-lectin conjugated) dissolved in TBS (20 μg/ml): ConA (Mannose - Man - and/or Glucose - Glc-), WGA (N-acetyl-D-glucosamine - GlcNAc - and/or sialic acid - NANA -) and DBA (N-acetyl-D-galactosamine - GalNAc -). After three washes in TBS, peroxidase activity was visualized with TBS containing 0.05% 3,3'-diaminobenzidine-tetrahydrochloride and 0.015% hydrogen peroxide. Sections were washed in running taper water for 10 minutes, dehydrated, cleared and mounted in Eukitt. The following controls were performed for the lectin staining: 1) substitution of lectin-HPR conjugates by TBS and 2) pre-incubation of the section with sugar inhibitors (Methyl-α-Man, D-GlcNAc and D-GalNAc).

Results

The skin of the gilthead seabream, *Sparus aurata* was composed of a cuticle, epidermis, dermis and hypodermis. The stratified squamous epithelium of the epidermis (Fig. 1A) is constituted by epidermal germinative or basal cells, epithelial cells and mucous or goblet cells (Figs. 1-4). Mucous cells were more numerous in tail and fins than in corporal skin. Clavate cells were not observed in seabream skin. The Bromophenol Blue reaction (general proteins) was evident in epithelial and germinative cells, and this reaction was weak or negative in mucous cells. Reductor groups were obseved in cuticle and in mucous cells of the seabream skin (Table 1).

Some mucous cells, specially those present in tail and fins, were positive to PAS and Alcian Blue, pH 0.5 and 2.5, and negative to Alcian Blue pH 1. PAS reactivity (Fig. 4C) was increased after saponification process suggesting the presence of acetylated sialic acid. Sialic

acids were also evidenced in mucous cells (corporal skin, tail and fins) with WGA (GlcNAc and/or NANA), because a slight decrease in WGA staining (Fig. 3B) and a decreased alcianophilia (Alcian Blue pH 2.5), after neuraminidase treatment, was observed in these cells. Mucous cells present in tail and fins contained variable amounts of acid glycoproteins weakly sulphated and carboxylated. Acetylated sialic acid, GlcNAc, GalNAc, Man and/or Glc residues were components of the mucus secretion of the *Sparus aurata* skin (Fig. 3; Table 1). Moreover, in cuticle and goblet cells, after sialidase treatment, an increased DBA staining was evidenced, suggesting the presence of sialic acid-GalNAc residues (Table 1).

In general, histochemical distribution of carbohydrates, proteins, and glycoproteins containing different saccharide residues, was similar in skin of healthy and of lymphocystis infected *Sparus aurata* specimens. However, goblet cell number and their sialic acid glycoproteic content was more copious in the skin of *Sparus aurata* with external lymphocystis disease sypmtoms.

The acidophilic hyaline capsule developed in mature lymphocystis-infected cells (Fig. 2) was diastase-PAS and PAS positive (Fig. 2B), indicating the absence of glycogen and the presence of reactive hexoses or deoxyhexose-rich glycoproteins, which were sulfated and carboxylated (Table 1). Hyaline capsule reacted strongly with Alcian Blue, pH 0.5 and 2.5, and weakly with Alcian Blue pH 1. Intracytoplasmic inclusions and granular cytoplasm of the mature lymphocystis-infected cells reacted with Con A (Fig. 4A) and they were unreactive with WGA (Fig. 3A) and DBA (Table 1). PAS reactivity in the granular cytoplasm and within intracytoplasmic inclusions was increased after saponification process (Fig. 4B). In the mature lymphocystis cells, the peripheral cytoplasm around hyaline capsule reacted strongly with Con A (Fig. 4A), the granular cytoplasm being unreactive with WGA (Fig. 3A) and weakly positive with DBA (Table 1).

Discussion

The skin of the gilthead seabream, *Sparus aurata* shows its general morphology similar to other species with scales (Roberts, 1978; Fouda, 1979). The epidermis is a stratified squamous epithelium covering the corporal surface. The epidermis is usually thicker in species like eel, *Anguilla* sp., with negligible scale cover and with numerous mucous cells (Roberts, 1978; Sarasquete et al., 1989) and consists of a tightly condensed layer of Malpighian cells with the basal cells showing columnar orientation and above these a transition to horizontal flattening; mucous cells are interspersed and appear as spheres opening to the surface within the upper zone. Goblet cells usually originate in the middle layers of the epidermis; they increase in size and elaborate secretions, mainly glycoproteins, as they approach the surface (Roberts, 1978).

In different species of fishes, the skin mucus is composed of mucopolysaccharides that contain sulphated and/or carboxyl radicals (Roberts, 1978). In fish, mucous secretion present interespecific differences. In *Pleuronectes platessa*, goblet cells contain sulphated

Table 1. Histochemical results in the skin and lymphocystis-infected cells of gilthead seabream *Sparus aurata* L.

TECHNIQUE	CUTICLE	EPITH	GERM	MUCO	LYMPHOCYSTIS		
					Cyt	Inc	Cap
Schiff (free aldehydes)	0	0	0	0	0	0	0
PAS (glycoconjugates with 1,2 glycols or amino-ol)	1	1	1	1-2	1	0	3
Diastase/PAS (glycogen)	1	0-1	0-1	1-2	0-1	0	0
KOH/PAS (Reactivity of acetylated hydroxyl and methylated carboxyl groups)	1-2	1	1	2-3	1-2	0-1	3
Alcian blue 2.5 (Carboxylated groups)	1-2	1	1	0-1	0	0	3
Neuraminidase or Chloridric hydrolysis/Alcian Blue 2.5 (Sialic acid)	1	1	1	0	0	0	2-3
Alcian blue 0.5 (Sulphated groups strongly ionized)	0	0	0	0-1	0	0	3
Alcian blue 1 (Sulphated groups (weakly ionizated))	0	0	0	0	0	0	1
WGA (GlcNAc/NANA)	3	0	0	3	1	0	0
Neuraminidase/WGA	1	0	0	1	1	0	0
Con A (Man/Glc)	3	2	2	0-2	1	1	0
DBA (GalNAc)	0-1	1	1	1	1	0	0
Neuraminidase/DBA	1	1	1	1-2	1	0	0
Bromophenol blue (General proteins)	0	2	2	0-1	1	1	1
Ferric-Ferricyanide-FeIII (Cysteine groups)	1	1	1	0-1	1	1	1
Tioglycollate/FerricFerricyanide-FeIII (Cystine groups)	2	1	1	2-3	1	2	2

Results are based on a subjectively estimated scale ranging from 0 to 3 with 0 being unreactive and 3 strongly reactive. Cap: Hyaline capsule; Cyt: cytoplasm; EPITH: epithelial cells; GERM: germinative or basal cells; In: intracytoplasic inclusions; MUCO: mucous or goblet cells.

groups (Fletcher and Grant, 1968), in other species they contain glycoproteins rich in sialic acid and/or sialosulphomucins (Askawa, 1970; Harris et al., 1973; Sarasquete et al., 1989; Illana, 1993).

In *Sparus aurata* skin, mucous cells, specially those of tail and fins were stained with PAS and diastase-PAS and weakly with Alcian Blue pH 0.5 and 2.5. PAS reactivity of the mucous cells was increased when sections were previously treated with KOH. These results, as well as, the decreased reactivity after neuraminidase treatment previously to WGA stain, could suggest the presence of acetylated sialic acid (C8, di or tri acetylated sialic acid) (Pearse, 1985), as well as the absence of glycogen (diastase-PAS positive) such as was evidenced in other species (Bullock et al., 1976; Sarasquete et al., 1989). Proteins were not detected in mucous cells of *Anguilla anguilla* and *Merlangius merlangius* (Bullock et al., 1976; Sarasquete et al., 1989). However, the Bromophenol Blue technique (general proteins) and PAS reaction were positive in some mucous cells of the *Sparus aurata* skin. According to Harrison et al. (1987) variability in staining within a

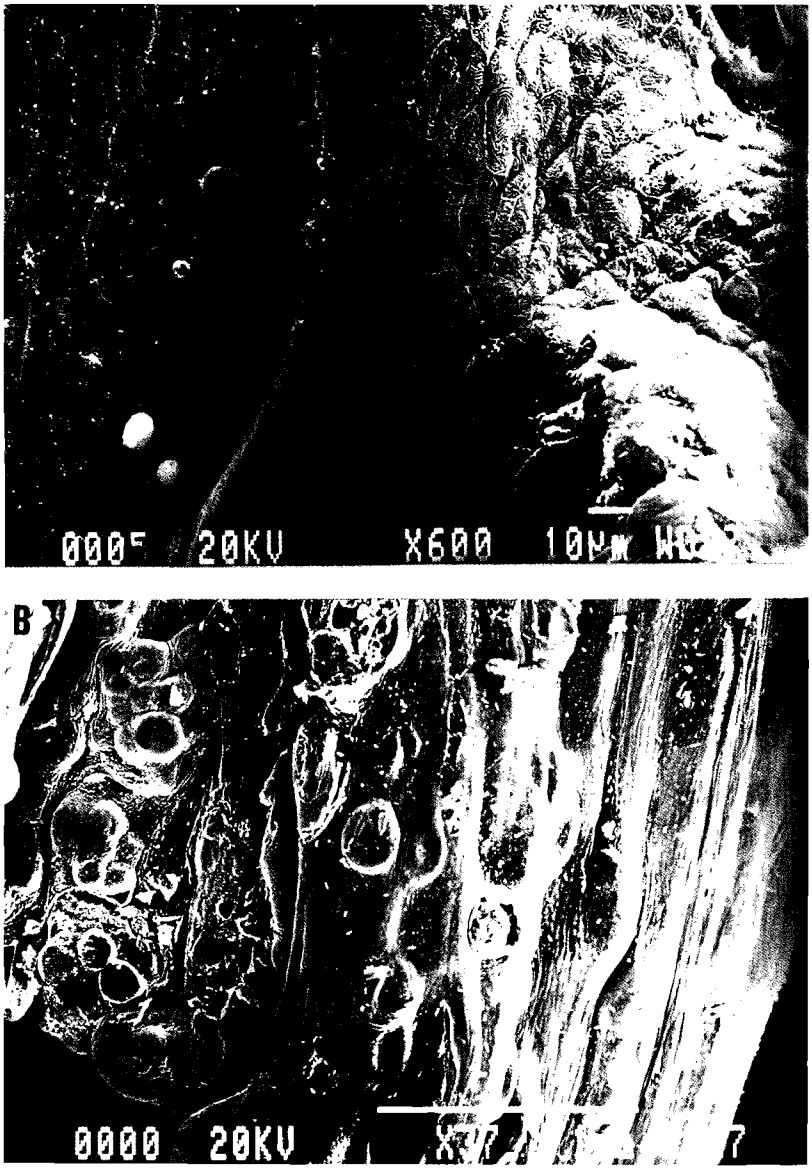

Fig. 1. Scanning electron microscopy of *Sparus aurata* L. skin. **A.** Surface of corporal skin showing a stratified squamous epithelium and mucous cells. **B.** Aspect of the viral lymphocystis disease in the skin of an infected specimen.

Fig. 2. Histological sections of skin with lymphocystis-infected cells. **A.** Haematoxylin-V.O.F. Gutiérrez. **B.** PAS-V.O.F. Gutiérrez. b: basal or germinative cells; c: connective fibroblasts (hyperplasia): e: epidermis; ep: epithelial cells; h: hyaline capsule; i: intracytoplasmic inclusions; ie: inflamatory epithelioid cells; m: melanophores; n: nucleus; nu: nucleoli. x 125

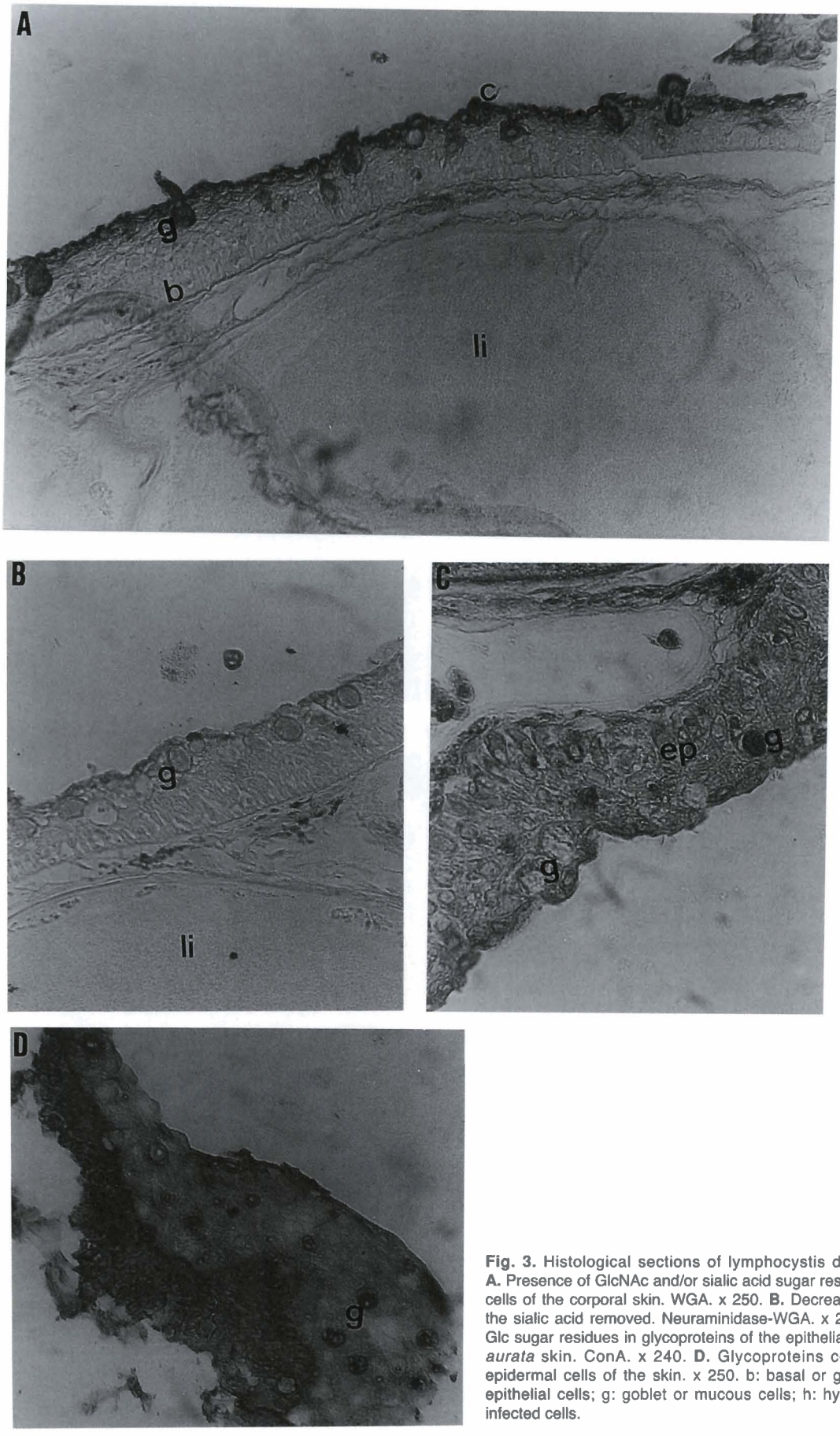

Fig. 3. Histological sections of lymphocystis disease in *Sparus aurata* skin. **A.** Presence of GlcNAc and/or sialic acid sugar residues in the cuticle and mucous cells of the corporal skin. WGA. x 250. **B.** Decrease in WGA staining related with the sialic acid removed. Neuraminidase-WGA. x 250. **C.** Presence of Man and/or Glc sugar residues in glycoproteins of the epithelial cells and cuticle of the *Sparus aurata* skin. ConA. x 240. **D.** Glycoproteins containing GalNAc residues in epidermal cells of the skin. x 250. b: basal or germinative cells; c: cuticle; ep: epithelial cells; g: goblet or mucous cells; h: hyaline capsule; li: lymphocystis-infected cells.

given cell could be attributed to a temporal sequence of biosynthesis of the mucous secretion. Byosynthesis of mucin glycoconjugates includes at least two post-transcriptional modifications to the secretory protein; firstly glycosylation of the protein followed by modifications to the sugar moiety (Phelps, 1978). Those cells that did not stain with PAS contained only proteins; mucous cells staining with PAS could be related to the stage when the cell was producing mainly glycoproteins: these cells would stain with Alcian Blue when the glycoproteins had been carboxylated and the presence of sulfated glycoproteins would coincide with the stage when the sulfated groups had conjugated to the glycoprotein (Els and Hennerberg, 1990). The presence of cystine bridges in cuticle and mucous cells of the *Sparus aurata* skin, could be related with its glycoproteic nature, as was suggested by Sarasquete et al. (1996) in other tissues of fishes.

Sialic acid has been used to estimate the degree of skin mucification in different species (Lemoine and Olivereau, 1971; Harris et al., 1973; Pickering, 1974). Arillo et al. (1979) pointed out that the gill sialic acid was an index of environmental stress. Sialic acid has been observed in mucous cells of *Sparus aurata* gills

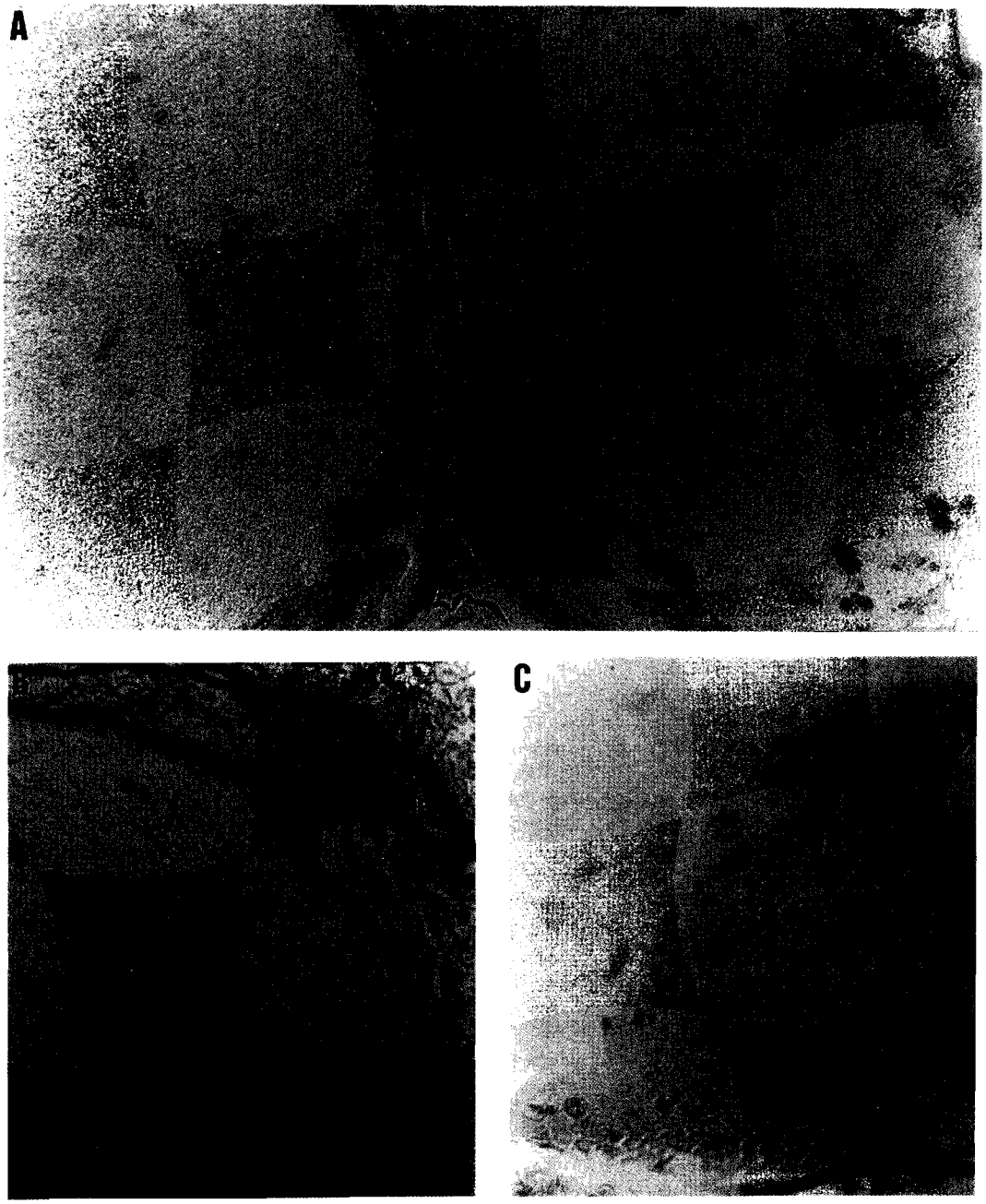

Fig. 4. Histological sections of lymphocystis-infected cells in *Sparus aurata* skin. **A.** Intracytoplasmic inclusions and granular cytoplasm of the mature lymphocystis- infected cells containing glycoproteins with Man and/or Glc residues. Unreactive hyaline capsule . ConA. **B.** Presence of acetylated sialic acid glycoconjugates in the granular cytoplasm and within intracytoplasmic inclusions developed in the mature lymphocystis infected cells. Saponification-PAS. **C.** Goblet cells containing glycoconjugates with 1,2 glycol or amino-ol radicals. c: cytoplasm of lymphocystis-infected cells; e: epidermis; ep: epithelial cells; g: goblet or mucous cells; h: hyaline capsule; i: intracytoplasmic inclusions. x 250

(personal observation). Illana (1993) histochemically showed the presence of sialic acid, GlcNAc, as well as Man and/or Glc sugar residues in glyco-proteins of goblet cells and cuticle of *Halobatrachus didactylus* and *Anguilla anguilla* skin. According to this author, goblet cells and cuticle of *Halobatrachus didactylus* failed to bind DBA, suggesting the absence of GalNAc residues. In *Sparus aurata*, the results obtained by conventional mucin histochemistry and by using lectins could suggest that WGA-reactivity in the epidermal goblet cells was due to GlcNAc and sialic acid sialic acid residues.

On the other hand, lymphocystis is a chronic viral disease reported in captive and wild marine and fresh water fish (Amin, 1979; Paperna et al., 1982). In *Sparus aurata*, during the development of the lymphocystis disease, proteins rich in different aminoacids were observed in the cytoplasm, nucleus/nucleoli, intracytoplasmic inclusions and hyaline capsule (González de Canales et al., 1996). Mucoproteins, glycoproteins or acid mucopolysaccharides (sulphated and carboxylated) were reported in the hyaline capsule of lymphocystis-infected cells of different fish (Nigrelli and Ruggieri, 1965; Howse and Christmas, 1978; González de Canales et al., 1996). In *Sparus aurata*, the hyaline capsule of the mature lymphocystis infected cells is composed of sulphated-sialoglycoproteins (González de Canales et al., 1996) and this capsule was unreactive with WGA, ConA and DBA lectins. Defendi and Gasic (1963) have shown that the main substances for a positive Hale's reaction in their *in vitro* virus-infected cells were sialomucins, whose carboxylic groups were responsible for the binding of ferric ions.

Intracytoplasmic inclusions and granular cytoplasm of the *Sparus aurata* lymphocystis-infected cells, contained glycoproteins with Man and/or Glc residues. These basophilic inclusions are composed of an electron-clear DNA or DNA-protein medulla; when lymphocystis-infected cells are mature, these inclusions become surrounded by icosahedral virus particles (Walker, 1965). In the intracytoplasmic inclusions developed in *Sparus aurata* lymphocystis infected cells, Bromophenol Blue reaction (general proteins) was positive and PAS reactivity was weakly positive after saponification process, suggesting the presence of acetylated hydroxyl and/or carboxylated groups. Bromophenol Blue reaction (general proteins) and Tioglycollate-Ferric-Ferricyanide-FeIII technique (cisteine and cystine) were positive in these intracytoplasmic inclusions. According to Rademacher et al. (1988), viral particles possess carbohydrate binding proteins which can interact with sugar structures on the surface of cells which the pathogen organisms associate.

In conclusion, the present results are taken to substantiate that GlcNAc, NANA, GalNAc and/or Man and/or Glc, are the most common carbohydrate residues in cuticle and mucous cells of the *Sparus aurata* skin. The hyaline capsule, developed in mature lymphocystis-infected cells, is composed of sulphated and carboxylated groups, but did not show any affinity towards ConA, DBA and WGA, suggesting the absence of Man, Glc, GlNAc and GalNAc residues in the carbohydrates involved. Man and/or Glc residues are present in the intracytoplasmic basophilic inclusions and in the granular cytoplasm of the lymphocystis-infected cells.

This study will be continued by the application of other techniques, such as ultrastructural studies, antibodies, as well as experimental viral inoculation in other species.

Acknowledgements. The authors are grateful to D. Alfonso Vidaurreta for supplying the fish and to Dña Isabel Viaña and D. Agustin Santos for valuable technical assistance. This work was supported by the Comision Interministerial de Ciencia y Tecnología from Spain (MAR95-1949-C02-02).

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Accepted April 29, 1997