Immune complex-mediated glomerulopathy in *Barbus graellsi* infected with *Myxobolus Spp*

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**Summary.** Membranous glomerulonephritis caused in *Barbus graellsi* by myxosporidian infections have been studied by electron microscopy and immunoelectron microscopy techniques.

This study indicates that Myxosporidian infection produces a chronic severe aggression. Spores reach the spleen, the kidney and the liver, where they are trapped and phagocyted by Melano Macrophage Centres. Consequently, the commencement of an immunological response to myxosporidian is evident. Our results show the presence of immunodeposits in the basement membrane of the glomeruli, suggesting that they might initiate glomerulonephritis. The lesion was markedly similar to immune complex-mediated glomerulonephritis disease in higher vertebrates.

**Key words:** *Barbus graellsi*, *Myxobolus Spp*, Glomerulonephritis, Immuneelectron microscopy

**Introduction**

Myxosporidian infections are widespread in marine and freshwater fish and they have variable pathogenic significance. Nigrelli and Smith (1938) were the first to recognize the inflammatory reaction of fish to myxosporidian infections. Recently, Roger and Gaines (1975), Needham and Wootten (1978) and Dykova and Lom (1988) have reviewed the pathological changes caused by myxosporidian.

The most commonly reported lesions of the kidney due to the presence of myxosporidians are either changes of renal interstitium, e.g. Proliferative Kidney Disease (PKD) (Clifton-Hadley et al., 1984, 1987) or changes of renal tubule epithelium, ranging from distrophy to necrosis (Copland, 1983; Molnar and Kovacs, 1986; Dykova and Lom, 1988), but there are few reports of the parasite causing glomerulonephritis in fish (Meyer and MacPherson, 1985). Consequently, we have paid special attention to the study of the membranous glomerulonephritis which was observed in the infected fish but not in the control fish.

It is known that in mammals a common trait observed in renal lesions resulting from protozoan infection is the thickening of the glomerular membrane (Costa et al., 1991). This lesion has rarely been documented in fish. In this report we present an electron microscope study of glomerulonephritis associated with electron-dense deposits in the glomeruli of *Barbus graellsi* infected with myxosporidians. We have carried out immuneelectron microscopic studies in order to identify the molecules found in these deposits since the characterization of the molecules found in the basement membrane of the glomeruli can aid the understanding of why they accumulate in the renal structures and how they cause renal injury.

**Materials and methods**

**Fish**

We examined 30 *Barbus graellsi* which were caught from the river Piedra in Zaragoza, Spain. They were approximately 27 cm (23 to 29.5 cm) in length and they were sacrificed in the laboratory by cervical dislocation.

**Parasitological investigation**

After necropsy, the presence of spores and trophozoites of *Myxobolus Spp* was confirmed by fresh squash preparations. Smears of fresh material were made and stained by Giemsa and/or Zielh-Nielsen from pieces of the kidney, liver and spleen from each fish, and were examined for the presence of spores and trophozoites of *Myxobolus Spp*.

Samples of infected fish were processed for histological study and tissues from non-infected fish were also examined and acted as control.

**Light microscopy**

For the purpose of histological examinations, tissue
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was fixed in 10% formal solution, embedded in paraffin, sectioned at 5 μm and stained with Hematoxylin-Eosin.

Routine electron microscopy

Tissue samples for electron microscopy were fixed in 2.5% buffered glutaraldehyde for 90 minutes, washed in Millonig buffer and dehydrated in ethanol. Fixed tissue samples were cleaned in propylene oxide and embedded in Epon-Araldite (1:1). Semithin (1 μm) and ultrathin (40-60 nm) sections were made, using an LKB ultramicrotome, and stained with toluidine blue, uranyl acetate, and lead citrate. Ultrathin sections were studied by transmission electron microscopy.

Immunoelectron microscopy

The kidney samples which were to be examined by electron microscopy, after being reacted with anti-Ig antibodies, were fixed in 0.5% buffered glutaraldehyde for 60 minutes. After washing in buffer, fixed samples were embedded in Durcupan water soluble Kit (Biorad). The ultrathin sections were incubated with normal pig serum for 30 minutes. After washing in phosphate-buffered saline (PBS), each section was incubated with the primary antibody (1:20, 12h, sheep anti-carps Ig, Biochrom). After washing in PBS, each section was incubated with the secondary antibody (1:150, 4h, rabbit anti-sheep Ig, Sigma), followed by biotin-labelled tertiary antibody (1:250, 30 minutes, ABC Kit PK-4001 Vector lab.). After washing, ABC complex (ABC Kit PK-4001 Vector lab.) was applied for 45 minutes. Enzyme reaction was developed with DAB-H₂O₂ solution (20 mg of 3',3'-diaminobenzidine in 100 ml of 0.05M tris-HCl buffer, pH 7.6, containing 0.005% H₂O₂) for 5 minutes and rinsed in PBS. Thereafter, the ultrathin sections, doubly stained with uranyl acetate and lead citrate, were examined by transmission electron microscopy.

Negative controls were performed by treating all sections with immunoglobulin fraction of non-immune sheep serum and rabbit serum as substitute for the primary and secondary antiserum. None of these control sections were immunostained.

Results

Viscera squash

In infected fish mature spores of Myxobolus Spp

Fig. 1. Myxosporian spores in a melan-macrophage centre (arrowhead) and isolated (arrow). Spleen fresh smear. Bar= 25 μm.

Fig. 2. Melano-macrophage centre in the liver interstitium containing engulfed myxosporian spores (arrows). Reticular cells (arrowheads). Bile canaliculus (*). Portain vein (V). Toluidine blue. Bar= 30 μm.
could be seen in the fresh squash made from the kidney, liver and spleen. Mature spores could be seen in the Melano Macrophage Centres (M-MCs) and also isolated (Fig. 1).

Liver

An increase in size and number of M-MCs was evident in the liver interstitium of infected fish. Most of M-MCs which contained spores were observed close to the branches of the portal vein (Fig. 2). The hepatic cells and the bilis canaliculus appeared normal.

Kidney

M-MCs with spores, showing different levels of cellular degradation (Fig. 3), were observed close to the vascular system of the kidney. Spores were found concentrated at these centres. Electron microscopy showed M-MCs to consist of phagocytes containing degenerated spores, surrounded by a continuous layer of flat reticular cells (Fig. 3).

In infected fish, the renal tubule showed hyaline droplet degeneration of the epithelial cells (Fig. 4). Coarse electron-dense granules appeared in the cytoplasm (Fig. 5). The size of the granules was not

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**Fig. 3.** Melano-macrophage centre in the kidney interstitium with spores (*) showing different levels of cellular degradation. Melano-macrophages (M). Reticular cell (R). Bar= 7 μm.

**Fig. 4.** Renal glomerulus showing capillary loop thickening (arrowheads) without mesangial proliferation. Mesangial stalk (*). Renal tubule showing both hyaline droplet degeneration of the epithelial cells and luminal occlusion by toluidine blue-positive materials (star). Toluidine blue. Bar= 30 μm.
uniform. Moreover, occlusion of both the proximal and the distal segments of the renal tubule, by the accumulation of certain materials in the lumen, was observed (Fig. 4). These materials were stained with eosin and toluidine blue. Some of the renal tubules showed focal infiltration of mononuclear cells which could be identified by electron microscopy as lymphocytes (Fig. 5). The lymphoid and haematopoietic tissue appeared normal.

Capillary loop thickening without mesangial proliferation was observed within the glomeruli (Fig. 4). A moderate and irregular thickening of the glomerular basement membrane containing electron-dense deposits was observed by electron microscopy (Fig. 6). Electron-dense deposits were found in various localizations within the same glomerulus; namely at: i) the subendothelial surface; ii) the intramembranous surface; and iii) the subepithelial surface. These deposits were observed either as large electron-dense deposits (Fig. 6A) or as annuli profiles (Fig. 6B). These annuli were 70-80 nm in diameter and frequently contained a central dense spot of material within the lucent interior.

Analysis by immunoelectron microscopy revealed a diffuse accumulation of immunoglobulin in both types of electron-dense intramembranous deposits (Fig. 7).

In non-infected fish, glomeruli and renal tubule appeared normal.

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**Fig. 5.** Part of a renal tubule showing hyaline droplet degeneration of the epithelial cells. Coarse electron-dense granules appeared in the cytoplasm. Epithelial cells (E). Lymphocytes (L). Bar= 10 μm.

**Fig. 6.** A. Glomerular capillary loop showing typical changes of membranous glomerulonephritis as greater thickness of basement membrane and subendothelial (thick arrow), subepithelial (arrow) an intramembranous (arrowhead) deposits. Erythrocyte (E). Bar= 6 μm. B. Part of a glomerular capillary loop showing electron-dense deposit as annuli profile. Bar= 1 μm.

**Fig. 7.** Part of a glomerular capillary loop showing electron-dense deposits associated with Ig. Electron-dense deposits can be seen as large deposits (A) or as annuli profiles (B). Bowman’s capsule (arrows). Foot processes of the podocytes (arrowheads). Erythrocyte (E). A; bar= 5 μm. B; Bar= 0,5 μm.
Zapata, These authors describe M-MCs as aggregations of myxosporidian infections. evaluation of fish health as a function of M-MC number commencement of an immunological described for other teleosts (Zapata and Cooper, 1990). material from circulation with the vascular system and surrounded by a since M-MCs have a role in the trapping of antigenic material from circulation (Ellis, 1980; Herraz and Zapata, 1986), we conclude that the concentration of spores within the M-MCs may indicate the commencement of an immunological response to myxosporidian infections.

Ziegenfuss and Wolke (1991) have proposed the evaluation of fish health as a function of M-MC number and/or size in the liver, since M-MCs are formed more slowly and in fewer number in the liver than in the spleen and kidney. The findings of the present study, showing M-MCs in large number and size in liver, suggest that myxosporidian infection is a chronic severe aggression.

In contrast to a mild host response caused by myxosporidian in both the spleen and liver, the kidney showed more serious lesions. Infected fish presented a membranous glomerulonephritis showing subendothelial, subepithelial and intramembranous electron-dense deposits without mesangial proliferation.

The pathogenesis of fish glomerulopathies is poorly understood. Our results show the presence of immunodeposits in the glomeruli of infected fish suggesting that they might initiate glomerulonephritis, as proposed by Ferguson et al. (1982) and Meyers and MacPherson (1985). Winter and Majid (1984) have proposed that, in mammals, antigen-antibody reaction develops glomerular lesion in both nephrototoxic and immunocomplex glomerulonephritis. Therefore, it can be assumed that immunodeposits located in glomerular lesions, which were observed in infected fish, are immune complexes (IC). IC unrelated to the glomerular basement membrane are formed elsewhere in the body and become trapped in the glomeruli. Localization of IC in the glomeruli is followed by the proliferation of mesangial cells and the thickening of the basement membrane (Winter and Majid, 1984). The mechanism by which these IC are retained in the kidney remains unsolved. It is known that, in mammals, mesangial cells are mononuclear phagocytes and possess receptors for IC and complement (Tizard, 1987). However, the functional characteristics of these cells in fish need to be elucidated. Germuth and Rodriguez (1973) and Muller-Peddinghans and Trautwein (1977) have suggested that in mammals, small IC are able to penetrate the basement membrane and that large IC are trapped subendothelially, they are then phagocyted by mesangial cells and give rise to proliferative mesangial lesions. Histological lesions in the glomeruli of the infected fish consisted of patchy thickening of the glomerular membrane without mesangial proliferation. Therefore our results, showing immunodeposits within the basement membrane, in conjunction with the finding that the only immunoglobulin class produced by teleost fish is similar to IgM (Clem and MacLean, 1975; Warr, 1983), suggest that in fish large IC are able to penetrate the basement membrane without mesangial proliferation.

Annuli-like deposits within the glomerular basement membrane have been identified by Dales and Wallace (1985) as autoantibodies bound to nuclear pore complexes. Our results show structures associated with Ig whose ultrastructural characteristics were similar to the deposits as described by the aforementioned authors. Therefore, we conclude that autoantibodies in the form of IC give rise to membranous glomerulonephritis in myxosporidian infections.

The development of circulating IC in response to an antigen is a common consequence of many infection processes. In the majority of instances these IC are removed from the circulation by phagocytic cells. Problems arise when there is a persistent antigenemia or when there is a predisposition to produce precipitating antibodies. In such cases, IC can be deposited in vascular endothelium and basement membranes (Gorman and Halliwell, 1989). However, immunodeposits are not uncommon in apparently healthy animals (Tizard, 1987). In the present study, it was exclusively infected fish which showed immunodeposits in the glomeruli. From this observation, it is evident that the extent of IC deposition is directly related to the presence of myxosporidian infections. In mammals a common trait observed in renal lesions resulting from protozoan infections is the presence of immuno-deposits occurring in a granular pattern (Sartori et al., 1991). Hence, the association of IC-mediated glomerulopathy in myxosporidian infections is not surprising.

Glomerular changes induced by IC adversely affect renal filtration and permeability. Therefore, both the occlusion of the renal tubules and the hyaline droplet degeneration of the epithelial cells are presumed to be linked to the malfunctioning of the glomerulus, but not to a direct pathological effect of myxosporidian infections.

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