NOTE

Histopathology of a herpesvirus infection in larvae of Japanese flounder *Paralichthys olivaceus*

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ABSTRACT: Histopathological lesions associated with a herpesvirus infection in Japanese flounder *Paralichthys olivaceus* larvae are described from seed production facilities in Japan. Experimentally and naturally affected flounder larvae had epidermal hyperplasia on the fins and skin and showed vacuolar degeneration of Malpighian cells that progressed to necrosis in advanced stages of the infection. The Feulgen reaction for DNA was positive only in the cell nuclei, and neither inclusion bodies nor syncytia formation was observed in any epithelial cells. Except for some minor renal degeneration, no remarkable histological changes or herpesvirus particles were observed in the gills or internal organs. Based on the histopathological findings, the name viral epidermal hyperplasia of flounder larvae is proposed for this disease.

Seed production of the Japanese flounder *Paralichthys olivaceus* has been practised intensively in public and commercial facilities in Japan since around 1980. A new disease characterized by fin opacity and associated with high mortality occurred in larvae of flounder in some hatcheries in Hiroshima Prefecture in 1985. Thereafter the disease was reported from other prefectures in Shikoku and Kyushu districts. Electron microscopy of affected epidermal cells and transmission experiments with tissue filtrates demonstrated that the disease was caused by a herpesvirus but attempts to culture the causative virus in fish cell lines have not been successful (Iida et al. 1989).

In this paper, we describe the disease process of this herpesvirus infection using histopathological examinations of flounder larvae specimens collected at regular intervals following their experimental infection.

Materials and methods. Naturally affected Japanese flounder larvae (8 to 9 mm in total length, 20 d after hatching), diagnosed as infected by the presence of the characteristic opaque fins, were taken from a commer-

cial hatchery in Hiroshima Prefecture. Some samples of these diseased fish were fixed for histopathological studies and the rest were frozen at -80°C and used as a source of virus for experimental infections. Healthy-appearing larvae (8.5 mm, 18 d) from ponds with no history for the disease were employed as test fish in infection experiments and as control fish for histological examinations.

Healthy larvae, kept in a 20 l tank at 20°C , were exposed to filtrates prepared by passing homogenates of naturally affected larvae through $0.45\text{ }\mu\text{m}$ membrane filters, as described previously (Iida et al. 1989). After virus exposure, 2 to 4 fish were collected daily for 16 d for histological examination. Fish with typical signs of the disease (opaque fins and skin) 14 d after exposure were submitted to transmission electron microscopy.

For light microscopy, naturally and experimentally infected larvae were fixed in Bouin's solution and embedded in paraffin wax. Sections were cut at 3 to $5\text{ }\mu\text{m}$ and stained with Haematoxylin-eosin (H & E), Azan, or Periodic Acid-Schiff (PAS). Samples fixed in Carnoy's solution were used for Feulgen staining.

For electron microscopy, fish were fixed in a 2.5 % glutaraldehyde/2 % paraformaldehyde mixture in phosphate buffer (0.1 M; pH 7.4) and post-fixed in 1 % osmium tetroxide. After embedding in Quetol 812, thin sections were prepared, stained with 1 % uranyl acetate and 1 % lead citrate, and examined with a JEOL 100CX electron microscope operating at an accelerating voltage of 75 kV.

Results. Light microscopy: In all examined specimens of naturally affected fish, the epidermal layer of the fins and skin was thickened over the entire body as a result of Malpighian cell hyperplasia (Figs. 1 & 2). Vacuolar degeneration of epithelial cell cytoplasm progressing to necrosis occurred in the superficial

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epidermis but not in the deeper region. Spherical epithelial cells were observed sloughing from the epidermal surface in the most advanced lesions. There was no hyperplasia or degeneration in branchial or intestinal epithelium and remarkable lesions were not seen in the internal organs, although some renal degeneration (see later) was noted.

The disease course was divided into 3 stages based on histological changes in experimentally infected fish:

(1) 0 to 4 d post-exposure: Fish did not show any external abnormalities during this period and except for one fish sampled at Day 4, which had slight epidermal hyperplasia on the fins and skin, there were no histopathological lesions in organs of 11 fish examined.

(2) 5 to 7 d post-exposure: Opaque areas began to appear grossly, particularly on the fins. However, significant mortality did not occur. Nine of 12 fish examined histologically had extensive foci of epidermal hyperplasia. Superficial epithelial cells containing nuclei with indistinct nuclear outlines and vacuolated cytoplasm (indicating the onset of degenerative changes) were observed only in a few samples.

(3) 10 to 16 d post-exposure: Mortality began to increase on Day 10 and by Day 16 all the fish had died. The typical external sign, epidermal opacity, was observed on the fins and skin of all fish. All 21 samples examined at the moribund stage had developed epidermal hyperplasia on the skin and fins. In the superficial epidermis of these fish, Malpighian cell degeneration with cytoplasmic vacuole formation was observed (Figs. 3 & 4). A mild cell infiltration in the dermis was seen in only one specimen (Fig. 3). There were no differences in the size and shape of epithelial cells or in basal membrane staining with Azan in infected and uninfected fish. PAS-positive cells were rarely observed in the hyperplastic epidermis of infected fish but secreted mucus was detected in some cells. The Feulgen reaction for DNA was positive only in the cell nuclei; inclusion bodies and syncytia formation were not observed in any epithelial cells (Fig. 3). Moreover, no pathological changes were observed in the branchial or intestinal epithelium (Figs. 5 & 6) or in other internal organs except for a slight focal hyalin droplet degeneration in the tubular epithelium of the kidneys of 9 fish.

Electron microscopy: Fig. 7 shows an epithelial cell from an affected fin at Day 14. The cell, fringed with microvilli, is shaped normally but distinct vacuoles are present in the cytoplasm. Hexagonal virus particles (120 nm average diameter) are evident in the nucleus

and enveloped particles (200 nm) are present in the cytoplasmic vacuoles. Nuclear membranes, desmosomes, and mitochondria with fine cristae structure were seen in epithelial cells containing numerous virus particles. Enveloped particles were also present in the extracellular space (Fig. 8). There were no virus particles in collagen fibers, dermal fibroblasts or in cells of the internal organs and gills.

Discussion. In the study, the major histopathological lesions in experimentally infected flounder larvae were confined to the epidermis of the fins and skin. Histopathological changes and virus particles were not observed in the gills or internal organs of affected fish, although minor hyalin droplet degeneration was noticed in the tubular epithelium of the kidney in some specimens. Throughout the course of infection the affected epidermis was most characterized by hyperplastic growth of the epithelial cells. This was followed by vacuolar degeneration of the cell cytoplasm. No nuclear or cytoplasmic inclusion bodies were observed. The pathological features found in experimentally infected flounder larvae were consistent with those observed in fish from natural epizootics.

The presence of nuclear membranes, desmosomes, and mitochondria with fine structure in cells containing numerous virus particles indicates that infected cells retain normal morphology even in advanced stages of infection and that cell necrosis occurs only in the terminal stages of the infection. Although the mechanisms that lead to larval mortality remain unknown, disturbance of epidermal integrity would be a significant lesion to larval fish. Considering that this herpesvirus targets epidermis, to cause hyperplasia, and the fact that only flounder larvae smaller than ca 10 mm in total length are highly susceptible to the virus (Masumura et al. 1989), we propose the name 'viral epidermal hyperplasia of flounder larvae' for this disease.

A similar disease caused by a herpesvirus has also been reported in hatchery-reared flounder larvae and juveniles in Mie Prefecture in Japan. Based on histopathological findings the disease was designated as epidermal necrosis (Miyazaki et al. 1989). Whether 'epidermal necrosis' and the present viral infection are the same disease is uncertain. Histologically, the diseases appear to be different. Miyazaki et al. (1989) reported that intranuclear and intracytoplasmic inclusion bodies were present in 'epidermal necrosis' but we detected no such bodies in 'viral epidermal hyperplasia'. Also, based on the dominant microscopic lesions found (epithelial necrosis versus epithelial

Figs. 1 to 6. *Paralichthys olivaceus*. Light micrographs of the epithelium of Japanese flounder larvae infected with a herpesvirus. H & E. Figs. 1 & 2. Normal (Fig. 1) and thickened (Fig. 2) epidermal layer of the skin and fins. (Bar = 0.1 mm). Figs. 3 & 4. Hyperplastic epidermis of the affected skin (Fig. 3) and fin (Fig. 4). Cytoplasmic vacuolar degeneration is observed in surface Malpighian cells. (Bar = 10 μ m). Figs. 5 & 6. Normal epithelium of gills (Fig. 5) and intestine (Fig. 6) in diseased fish. (Bar = 100 μ m)

Figs. 7 & 8 *Paralichthys olivaceus*. Transmission electron micrographs of an epithelial cell of Japanese flounder larvae infected with a herpesvirus. Fig. 7. Hexagonal virus particles (V) in the nucleus and enveloped particles (VE) in cytoplasmic vacuoles. D. desmosome; M. mitochondria; MV: microvilli. (Bar = 1 μ m) Fig. 8. Enveloped virus particles in the extracellular space. (Bar = 100 nm)

hyperplasia), the diseases appear to be different. It is possible, however, that the differences in the histopathology may be more apparent than real and that they reflect the fact that Miyazaki et al. (1989) examined only moribund fish. Firm conclusions as to whether the diseases are the same will likely only be possible if the causative viruses can be isolated and studied. Should the viruses prove to be identical, decisions will then have to be made on which of the 2 names proposed for the disease caused by the virus is more appropriate.

Epidermal hyperplasia associated with herpesvirus infections in fish have been reported in lake trout (Bradley et al. 1989, McAllister & Herman 1989), European eel (Békési et al. 1986), smelt (Anders & Möller 1985), golden ide (McAllister et al. 1985), northern pike (Yamamoto et al. 1983), sheatfish (Békési et al. 1984), walleye (Kelly et al. 1983), and turbot (Richards & Buchanan 1978). As in the flounder larvae, epidermal hyperplasia was the major lesion in the species listed

above. However, the histopathological features in the flounder larvae infection differed from those in the above species with respect to organ specificity, the lack of inclusion bodies, and the absence of giant cell formation. Moreover, because among several species of marine fishes tested only flounder larvae appeared to be susceptible to the present virus (Masumura et al. 1989), the causative virus may well represent a new fish herpesvirus.

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LITERATURE CITED

- Anders, K., Möller, H. (1985). Spawning papillomatosis of smelt, *Osmerus eperlanus* L., from the Elbe estuary. J. Fish Dis. 8: 233-235

- Békési, L., Horváth, I., Kovács-Gayer, E., Csaba, G. (1986). Demonstration of herpesvirus like particles in skin lesions of European eel (*Anguilla anguilla*). J. appl. Ichthyol. 2: 190–192
- Békési, L., Kovács-Gayer, É., Rátz, F., Turkovics, O. (1984). Skin infection of the sheatfish (*Silurus glanis* L.) caused by a herpes virus. Symposia Biologica Hungarica 23: 25–30
- Bradley, T. M., Medina, D. J., Chang, P. W., McClain, J. (1989). Epizootic epitheliotropic disease of lake trout *Salvelinus namaycush*: history and viral etiology. Dis. aquat. Org. 7: 195–201
- Iida, Y., Masumura, K., Nakai, T., Sorimachi, M., Matsuda, H. (1989). A viral disease in larvae and juveniles of the Japanese flounder, *Paralichthys olivaceus*. J. aquat. Anim. Health 1: 7–12
- Kelly, R. K., Nielsen, O., Mitchell, S. C., Yamamoto, T. (1983). Characterization of *Herpesvirus vitreum* isolated from hyperplastic epidermal tissue of walleye, *Stizostedion vitreum vitreum* (Mitchill). J. Fish Dis. 6: 249–260
- Masumura, K., Iida, Y., Nakai, T., Mekuchi, T. (1989). The effects of water temperature and fish age on a herpesvirus infection of Japanese flounder larvae, *Paralichthys olivaceus*. Fish Pathol. 24: 111–114
- McAllister, P. E., Herman, R. L. (1989). Epizootic mortality in hatchery-reared lake trout *Salvelinus namaycush* caused by a putative virus possibly of the herpesvirus group. Dis. aquat. Org. 6: 113–119
- McAllister, P. E., Lidgerding, B. C., Herman, R. L., Hoyer, L. C., Hankins, J. (1985). Viral diseases of fish: first report of carp pox in golden ide (*Leuciscus idus*) in North America. J. Wildl. Dis. 21: 199–204
- Miyazaki, T., Fujiwara, K., Kobara, J., Matsumoto, N., Abe, M., Nagano, T. (1989). Histopathology associated with two viral diseases of larval and juvenile fishes: epidermal necrosis of the Japanese flounder *Paralichthys olivaceus* and epithelial necrosis of black sea bream *Acanthopagrus schlegelii*. J. aquat. Anim. Health 1: 85–93
- Richards, R. H., Buchanan, J. S. (1978). Studies on *Herpesvirus scophthalmi* infection of turbot *Scophthalmus maximus* (L.): histopathological observations. J. Fish Dis. 1: 251–258
- Yamamoto, T., Kelly, R. K., Nielsen, O. (1983). Epidermal hyperplasias of northern pike (*Esox lucius*) associated with herpesvirus and C-type particles. Arch. Virol. 79: 255–272

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