Pathogenicity of Iridovirus from Japan and Thailand for the Red Sea Bream Pagrus major in Japan, and Histopathology of Experimentally Infected Fish

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The present study evaluated the pathogenicity of iridovirus from red sea bream Pagrus major and sea bass Lateolabrax sp. in Japan and one from brown-spotted grouper spawner Epinephelus malabaricus (GSIV: grouper spawner iridovirus) in Thailand to the red sea bream. The iridovirus from red sea bream in Japan caused 75% mortality by intraperitoneal (i.p.) injection with 10^{3.2} TCID_{50}/fish and 90% mortality by immersion infection with 10^{2.7} TCID_{50}/ml. Iridovirus isolated from imported sea bass caught in the South China Sea and then reared in Japan caused 100% mortality in red sea bream by i.p. injection with 10^{2.3} TCID_{50}/fish. Red sea bream had 55.6% and 55% cumulative mortalities by an i.p. injection of the spleen filtrates of GSIV from Thailand. The same histopathological changes as red sea bream iridovirus infected fish were observed in all of the experimentally infected red sea breams. The most characteristic changes were the appearance of large numbers of blast-like inflammatory cells throughout the circulatory system and the formation of markedly enlarged cells in the spleen, kidney, heart, liver, digestive tracts, pancreas, gills, swim bladder, choroid and choroidal rete of eyes, meninges, bone and musculature. Electron microscopy showed virions with an edge-to-edge diameter of 175 to 196 nm in all experimentally infected groups. Immunofluorescence positive reaction with the monoclonal antibody against red sea bream iridovirus was observed in all experimental groups.

Key words: iridovirus in Japan and Thailand, pathogenicity, histopathological comparison, serological comparison

An iridoviral disease was reported in an aquarium fish, chromide cichlid Etroplus maculatus, imported from Singapore into Canada in 1989.1) Histopathological changes included the appearance of ballooning degeneration of cells in the gills, kidney and intestine, and many blast-like cells throughout the circulatory system. A similar iridoviral disease called “Sleepy Grouper Disease” was reported in the cultured brown-spotted grouper Epinephelus taupina in Singapore in 1992.2) In Japan, iridovirus infection has spread in the western regions among cultured seawater fishes such as red sea bream Pagrus major, Japanese parrot fish Oplegnathus fasciatus, striped jack Caranx deliciatissimus and yellowtail Seriola quingernadiata, and has produced mass mortalities every year in the summer season since 1990.3-5) The isolates from different fish species in Japan were shown to be antigenically similar by the reactivities of monoclonal antibodies.6,7) In Thailand, two iridovirus infections were known in the brown-spotted grouper Epinephelus taupina.8,9) One is very similar to the diseases mentioned above.9) The virus affects wide age ranges, from fingeling to spawner, and infected fish displayed pale or dark coloration of the body surface, pale gills and splenomegaly. The characteristic histopathological signs were enlarged cells (9×11-14×20 µm) in the spleen, kidney, heart and digestive tract accompanying with the necrosis of the spleen and kidney. We referred to the iridovirus as a GSIV (grouper spawner iridovirus). The other,9) referred to as a grouper iridovirus (GIV), only infects fry and fingeling showing darkening in the tail and fin, and causes lysis in EPC (Epithelioma papulosum cyprini) and GF (grouper fin) cell lines. Histopathological studies on the GIV have not been reported yet. Although there is a large geographical distance between Southeast Asia and the Far East, the diseases (except GIV) show similarities in outbreak situations and clinical signs, histopathological and electron microscopic features in diseased fishes.1-3,8)

Miyata et al.10) found that iridovirus DNA from (i) red sea bream, (ii) sea bass Lateolabrax sp. which were captured in the South China Sea and which suffered an outbreak after being imported into Japan, and (iii) GSIV from Thailand were amplified by polymerase chain reaction (PCR) and concluded that the viruses had the same origin. To test whether these genetically similar iridoviruses can cause the same disease, we performed infectivity experiments in which red sea bream, which are the main species affected by this virus, were infected with iridoviral isolates from red sea bream, imported sea bass in Japan and GSIV from Thailand. Histopathological and electron microscopic observations, and immunofluorescence (IF) tests were conducted for all groups of experimentally diseased red sea bream.
Materials and Methods

Virus for Infectivity Tests
Iridovirus was isolated in Japan from red sea bream and imported sea bass. The iridovirus infected red sea bream (15-500 g) were collected at several fish farms in Mie prefecture in 1994 and 1995. The sea bass were caught in the South China Sea in their juvenile period, then transported to Mie Prefecture in February of 1993. Moribund sea bass (30-50 g) were recognized to have an iridovirus infection in August of 1993. Diseased fishes displayed splenomegaly and a pale coloration of the gills, kidney and liver. For the GSIV from Thailand, frozen spleens of iridovirus infected fish were transported by air to Japan for the experiments. The epizootics and pathological signs of brown-spotted grouper infected with GSIV were described by Danayadol et al.8)

Virus was isolated using the BF-2 (bluegill fry) and GF (grunt fin) cell lines and incubated for 14 days at 25°C. Fifty percent end-points (TCID50) were calculated using the method of Behren-Käber.11)

Experimental Infection
1) Infectivity of Iridovirus from Red Sea Bream for Red Sea Bream
Red sea bream were infected by intraperitoneal (i.p.) injection or by immersion. The first group of 20 red sea bream (14.7-24.0 g) was injected with 0.1 ml of MEM containing 104.2 TCID50/ ml of iridovirus from red sea bream. For the control, the same amount of MEM from the supernatant of an uninfected cell culture was injected. The second group of 20 red sea bream (4.2-6.4 g) was infected by immersion for 1 h in 2 l of sea water to which virus supernatant was added to give a virus concentration of 102.7 TCID50/ml. Control fish were exposed to water containing cell culture medium without virus.

Moreover, in order to reveal the progression of the disease, an immersion transmission experiment was done. Forty red sea breams (4.2-6.4 g) were exposed to bath challenge with 1025 TCID50/ml of the virus in containers with 3 l of sea water. Control fish were exposed to water containing cell culture medium without virus. Three fish were sampled at 24 h intervals for 13 days for histopathological examination.

2) Infectivity of Iridovirus from Sea Bass for Red Sea Bream
Twenty red sea bream (14.7-24.0 g) was injected with 0.1 ml of MEM containing 103.3 TCID50/ml of virus and one control group was injected with the same amount of MEM from the supernatant of the uninfected cell culture. The BF-2 and GF cells failed to culture the GSIV, apparently because the iridovirus from this fish causes very slight cytopathic effects. This may be caused by decreasing virus titer due to long storage (18 months) at -80°C and the difficulty of culture of the iridovirus in cell lines.3,4) Consequently, a filtrate of spleen homogenate was used for the infectivity test. The experiments were conducted twice. Twenty-seven (14.7-24.0 g) and 20 (4.2-6.4 g) red sea breams were inoculated with 0.1 ml of spleen filtrate containing GSIV. Twenty-seven and 20 control fish were injected with the cell culture supernatant without virus. At 21 days postinfection, all surviving fish were sacrificed.

All fish groups were held in 130 l aquaria receiving a flow-through supply at 27 to 29°C. Moribund and dead fish were processed for virus isolation, histopathological observations, electron microscopic observations and IF testing.

Histopathology and Electron Microscopy
Standard histological and electron microscopic procedures13) were used for moribund and dead fish in both of the natural outbreaks and the experimental infections.

Immunofluorescence Test
The monoclonal antibody (MAb) against red sea bream iridovirus, M10, was kindly provided by Dr. Nakajima. The MAb was tested with the spleen imprints of the moribund red sea bream from all experimental groups as described by Nakajima and Sorimachi.6)

Results

Infectivity Experiments
1) Pathogenicity of iridovirus from red sea bream for red sea bream
The cumulative mortality curves of the infectivity trials are shown in Fig. 1. In the injected fish, the first death occurred 10 days postinjection and cumulative mortality was

Fig. 1. Cumulative mortalities of red sea bream inoculated with the iridovirus from sea bass and red sea bream.
- • Intraperitoneal injection with 103.5 TCID50/ fish of the virus from red sea bream.
- △ Immersion infection with 102.7 TCID50/ ml of the virus from red sea bream.
- ○ Injection with 102.5 TCID50/ fish of the virus from imported sea bass.
- △ Control for group.
There was no mortality in the control group.

### 2) Pathogenicity of Iridovirus from Sea Bass for Red Sea Bream

The fish injected with $10^{2.3}$ TCID$_{50}$ of the virus showed 100% cumulative mortality by day 8 postinjection (Fig. 1). There was no mortality in the control group.

### 3) Pathogenicity of GSIV for Red Sea Bream

In the first experiment, 15 red sea bream out of 27 died during the infection trials. The cumulative mortality was 55.6% (Fig. 2). In the second test, 11 fish died and the cumulative mortality reached 55% by day 21 postinjection (Fig. 2).

### Histopathological and Electron Microscopic Features of Experimentally Infected Red Sea Bream

The same histopathological changes were observed in all red sea bream groups infected with iridovirus isolates from red sea bream and sea bass in Japan and Thai brown-spotted grouper. The most striking finding was the formation of enlarged cells (Fig. 3A) and abundant blast-like inflammatory cells throughout the circulatory system (Fig. 3B).

The enlarged cells usually had intensively basophilic and Feulgen positive cytoplasm, and an expanded nucleus with a prominent nucleolus (Fig. 3A). Electron microscopy revealed that the basophilic and Feulgen positive element of these cells was an inclusion body containing compactly packed iridoviral virions (Figs. 4A, 4B). Therefore, hereafter we refer to the enlarged cells as inclusion body bearing cells (IBC). There were also IBCs having a granular cytoplasm or markedly clear cytoplasm due to the release of the virions from the inclusion body (Fig. 3A).

Virus particles observed by electron microscopy had edge-to-edge diameters ranging from 175 to 196 nm (average 186 nm) in all experimentally infected groups (Fig. 4C).

In the spleen, large numbers of IBCs appeared in the splenic pulps and sheathed tissue. Diffuse necrosis appeared in the spleen, mostly in the pulps where reticular cells were affected, and were accompanied by haemorrhage (Fig. 3C). Necrosis was also observed in the walls of splenic arteries and veins. In electron microscopy the necrotic cells in the spleen and haematopoietic tissue showed diffuse multiplication of iridoviral virions in the cytoplasm. In the blood vessels, many blastic inflammatory cells of varied sizes were seen to be migrating mixed with unidentified cell debris (Fig. 3B). The haematopoietic tissue throughout the kidney was sometimes packed with IBCs accompanying the necrotic changes (Fig. 3A). The glomeruli had dilated, empty capillaries due to IBC emboli and exfoliation of necrotic endothelial cells. In severe cases, the IBCs were also observed in the corporules of Stannius. The liver had multiple discrete necrotic foci in the parenchyma. The IBCs were usually found in the vascular walls of the portal vein and hepatic vein where inflammatory cell infiltration had frequently occurred. The IBCs were abundant in the wall of the sinus venosus, and myocardium, endocardium and epicardium of the atrium, ventricle and bulbus arteriosus. Numerous inflammatory cells, approximately the same number as red blood cells, were observed with cell debris of necrotized cells in the lumen of the heart. Edema was found in myocardials. Sometimes the apex of the heart showed extensive haemorrhage. In the gills, many IBCs were found along the branchial arteries and the central venous sinus, in which inflammatory cells migrated, as well as in the interlamellar epithelia of the filaments. In some fish, hyperemia of lamellar capillaries, hyperplasia of the respiratory epithelium and necrosis of the pillar cells were also observed. In the esophagus, stomach and intestine, the lamina propria, submucosa and serous membrane contained inflammatory cells and IBCs. Especially, the red sea bream group infected by immersion had much larger numbers of inflammatory cells in the lamina propria than the groups infected by i.p. injection. The pancreas displayed inflammatory cells and IBCs around blood vessels with necrosis of acinar cells. In the eyes, inflammatory cells and IBCs appeared in the choroidal rete, choroid and iris. No pathological changes related to neurons in the brain were observed, but few inflammatory cells and IBCs were observed in the meninges and blood vessels. In the space within the bone, inflammatory cells and IBCs were observed around the blood vessels but no changes were observed in the cartilage or notochord (Fig. 3D). In severe cases, the epithelium of the swim bladder and rete mirabile also contained IBCs. The musculature showed severe inflammatory cell infiltration with few IBCs between the muscle fiber bundles (Fig. 3E). The thymus, skin, spinal cord and ganglion did not show histopathological changes.

In the periodic examinations of the fish infected by immersion, inflammatory cell reaction was evident in the intestinal tissue (Fig. 3F) from the 4th day after infection. A few inflammatory cells were also exhibited in the heart, head kidney and spleen on day 4. On day 7, inflammatory cells were observed throughout the circulatory system but no other histopathological changes were observed. The IBCs began to be observed in fish on the 5th day of infec-
Fig. 3A. The head kidney of red sea bream injected with iridovirus from imported sea bass. IBCs (inclusion body cells) with intensively basophilic (a), granular (b), or clear (c) cytoplasm. An expanded nucleus with a prominent nucleolus (small arrow). (H-E)

Fig. 3B. Interior of a blood vessel of the red sea bream injected with GSIV (grouper spawner iridovirus) sampled in Thailand. Several different sizes of inflammatory cells (arrows) are seen mixed with cell debris. (H-E)

Fig. 3C. The spleen of red sea bream injected with GSIV. Severe diffuse necrosis with IBCs formation occurs accompanied by haemorrhage. (H-E)

Fig. 3D. The bone of red sea bream injected with iridovirus from sea bass. Inflammatory cells and IBCs are seen in the space within the bone. (H-E)

Fig. 3E. The musculature of red sea bream injected with GSIV. Inflammatory cells have infiltrated in the musculature. (H-E)

Fig. 3F. The intestine of red sea bream which is 5 days after immersion infection with iridovirus from red sea bream. Infiltration of many inflammatory cells occurs in the lamina propria. (H-E)

Immunofluorescence Test

IF-positive cells, containing IBCs, were observed in all experimental groups. The spleen imprints of red sea bream infected with iridovirus in Japan and Thailand showed the same reactions to the M10 MAb against red sea bream.

Slight necrosis in the spleen, kidney and occasionally in the liver were observed from the 8th day.

Immunofluorescence Test

IF-positive cells, containing IBCs, were observed in all experimental groups. The spleen imprints of red sea bream infected with iridovirus in Japan and Thailand showed the same reactions to the M10 MAb against red sea bream.
Discussion

The results of the infectivity experiments indicated that the iridovirus isolates in Japan from red sea bream and imported sea bass were pathogenic to red sea bream. In the same way, the GSIV from Thailand was also pathogenic to red sea bream. The histopathological signs were mostly the same in all experimental groups of red sea bream and were also the same as those of naturally diseased red sea bream which we had studied. The MAb against red sea bream iridovirus reacted with the spleen imprints from all experimentally infected red sea bream.

Histopathologically, all infected fish displayed IBCs in spite of the different sources of the iridovirus isolates. The IBCs were the main characteristic sign of this iridoviral infection although previous reports described several different names, such as ballooned cells, \(^1\) heteromorphic balloon cell, \(^2\) enlarged cells\(^4\text{-}^7\text{,}^13\) or circumscribed bodies\(^2\) etc., which were not found in Epizootic Haematopoietic Necrosis\(^4\text{-}^8\). We named these cells inclusion body-bearing cells (IBC), indicating that the cells are specialized for efficient virus assembly in this infection. IBCs may play a role as a factory of virus multiplication. After large amounts of virus assembly occur in an IBC, the cell may lyse, and then enormous numbers of virus particles can be released rapidly. Moribund fish contained many IBCs in the gills and intestine which have direct contact with the external environment. In warm water, these tissues are easily lysed after the death of the fish. In this situation, even a slight physical press would cause tissue fragments containing IBCs to be separated from the lytic gills and intestine into the surrounding water. Therefore, the separated IBCs can spread innumerable virions into the water to transmit the virus among reared fish in the same cage. Thus, the occurrence of IBCs appears to be a very effective way to induce severe dissemination in a short time among fish.

As to virion size, Inouye \(et\ al\).\(^1\) and Danayadol \(et\ al\).\(^8\) reported considerably different virion size in different fish ranging 120–240 nm in diameter. We measured GSIV in Thailand, and iridovirus from red sea bream, imported sea bass and striped jack in the natural outbreaks in Japan, and experimentally infected red sea bream in this study. The virion size was in the range from 150 to 196 nm in edge-to-edge diameter in all samples. The size differences by reporters may be caused by different fixative conditions or different ways to determine size.

By periodic sampling, we found that inflammatory cells react very actively from the early stage of infection before
IBCs appear. Some of the virus infected inflammatory cells might develop into IBCs because the area of inflammatory cells in tissues in the early stage of infection coincided with the area of IBCs in moribund fish. The exact origin of the IBCs must be determined by further studies.

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References

4) K. Nakajima, and M. Sorimachi: Biological and physico-chemical properties of the iridovirus isolated from cultured red sea bream, Pagrus major. Fish Pathol., 29, 29-33 (1994).