Multiplication of Red Sea Bream Iridovirus (RSIV) in the Experimentally Infected Grouper

Epinephelus malabaricus

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ABSTRACT—The multiplication of red sea bream iridovirus (RSIV: KM99 from red sea bream) was investigated in the two groups of grouper Epinephelus malabaricus (average body weight, 7.7 g and 84.3 g) intraperitoneally inoculated with the virus at 28°C. Dead fish were observed from 8th day post-inoculation, and the cumulative mortality was 90% in fish of both sizes. Viral antigen-positive cells were detected with an indirect immunofluorescence test using a monoclonal antibody in the spleen and head kidney of the infected fish from 2nd day post-inoculation and in the liver and body kidney from 6th day post-inoculation. The number of positive cells in the spleen and head kidney was higher than that in the liver and body kidney. Transmission electron microscopy showed that the multiplication of the virus occurred in the enlarged cells in the fish at 4th day post-inoculation. In the dead fish, virus particles were found around the disrupted enlarged cells and small blood vessels. These findings suggest that the enlarged cells form primarily in the spleen and head kidney of the infected grouper as a result of viral multiplication and that the cells move to other organs via blood vessels and cause disruption by releasing progeny viruses.

Key words: viral multiplication, red sea bream iridovirus, Epinephelus malabaricus, immunofluorescence, TEM, RSIV, grouper

In recent years, seed production of a grouper, Epinephelus malabaricus, has been successful, and the seedlings are provided for commercial aquaculture in Okinawa Prefecture, a subtropical area of Japan. This grouper is an especially promising candidate to promote the fish aquaculture industry in this area. However, mortality of the cultured grouper has occurred since 1996 by red sea bream iridoviral disease (RSIVD), which was first reported in cultured red sea bream, Pagrus major, in western Japan as a systemic iridoviral infection (Inouye et al., 1992). RSIVD is an acute contagious disease among marine cultured fish in over 30 species (Inouye et al., 1992; Matsuoka et al., 1996; Kawakami and Nakajima, 2002). Experimental infection trials confirmed that this grouper was highly susceptible to RSIV (Sano et al., 2000). The characteristic histopathological changes in the infected grouper are the degeneration of the spleen and head kidney and the appearance of a basophilic enlarged cell (Sano et al., 2000), as in red sea bream infected with RSIV (Inouye et al., 1992). Degeneration of the liver, body kidney, digestive tract, and gill was not notable in the grouper.

A systemic iridoviral infection was also reported in the following grouper: E. tauvina in Singapore (Chua et al., 1994), E. malabaricus in Thailand (Danayadol et al.,...
1997; Kasornchandra and Khongpradit, 1997), and Epinephelus sp. in Taiwan (Chou et al., 1998). Danayadol et al. (1997) reported that the iridovirus in the grouper was genetically similar to RSIV, and another iridovirus in Thailand, GIV (Kasornchandra and Khongpradit, 1997), has different properties from RSIV (Nakajima et al., 1998). Although the relationship between the other two viruses in other countries and the RSIV in Japan is still undefined, systemic iridoviral infections are obstacles to the grouper culture in sub-tropical and tropical areas. Histopathological findings in RSIV-infected grouper are similar to those of other iridovirus infections in other countries (Sano et al., 2000). The disease process, including viral growth in infected fish, however, is not well understood in both the grouper and the red sea bream.

The present study was undertaken to provide a more detailed investigation of the multiplication and pathogenicity of RSIV in an infected grouper, E. malabaricus, mostly by means of an indirect immunofluorescence (IF) test and transmission electron microscopy (TEM) of the fish sampled at certain intervals after experimental intraperitoneal inoculation.

Materials and methods

Virus

RSIV Kumamoto isolate (KM99), which had been originally isolated from diseased red sea bream in 1996, was used. The virus suspension for experimental infection was prepared as reported previously (Sano et al., 2000). The virus titer was determined on the GF cell line (Clem et al., 1961) at 25°C.

Experimental infection and fish sampling

Two sizes of hatchery-reared grouper, E. malabaricus (average body weight, 7.7 g and 84.3 g), were intraperitoneally inoculated with the viral suspension at a dose of \(10^{4.2}\) TCID\(_{50}\)/fish. As a control, fish of each size were injected with minimum essential medium. Eighty fish inoculated with the virus and forty fish of control were divided into four and two aquariums, respectively. One aquarium per group was used for observation of daily mortality, and the other three aquariums of virus-inoculated group and one aquarium of control were prepared for sampling three fish from each at 2, 4, 6, 8, 10, and 14th days post-inoculation. The experimental fish were reared in aquariums supplied with seawater at 28°C for 14 days and fed with commercial diet. Dead fish were collected from the aquariums. The spleen, head kidney, liver, and body kidney were dissected from the sampled and dead fish, and these organs were divided into three portions for processing and conducting IF, light microscopy, and TEM examinations.

IF test for detecting the viral antigen

A portion of the sampled organs was smeared onto a 1 × 1 cm square area on a slide glass to detect the viral antigen positive cell by an IF test using the monoclonal antibody (M10) as described by Nakajima et al. (1995). The monoclonal antibody recognizes RSIV-induced polypeptide (Nakajima and Sorimachi, 1995).

Light microscopy

A portion of the organs was fixed in 10% phosphate-buffered formalin and processed into routine paraffin sections followed by hematoxylin and eosin (H & E) staining.

TEM

Another portion of the spleen and head kidney was fixed in 2.5% glutaraldehyde solution buffered with 0.1M phosphate buffer (pH 7.2) for 4 hours and post-fixed in 1% aqueous OsO\(_4\). It was then dehydrated through an ethanol series and embedded in Spurr resin (Polyscience). Ultrathin sections were stained with uranyl acetate and lead citrate prior to examination with a JEM-1010 electron microscope at 80 kV.

Immunohistochemistry

The head kidneys were dissected from two dead fish of the large fish group at 9th day post-inoculation. A small portion of each was fixed in acetone and dehydrated through an ethanol series and then embedded in Technovit 8100 (Kulzer) on ice. The specimens were stored in a refrigerator before making sections. Sections were treated with the monoclonal antibody M10 and peroxidase-conjugated anti-mouse-IgG goat IgG (Wako Pure Chemicals Industry), and the positive reactions were visualized in a diaminobenzidine solution followed by hematoxylin staining.

Results

Gross sign and mortality of experimental fish

In the two groups of each size, food consumption of the inoculated fish decreased drastically, and the fish cast mucoid and opaque faecals 6 days after inoculation. Moribund fish showed darkened body in color. Dead fish were recorded from 8th to 10th day post-inoculation in the inoculated group of large fish and from 8th to 11th day post-inoculation in the inoculated group of small fish, and cumulative mortality reached 90% in both groups (Fig. 1). No dead fish were observed in the control groups.

Detection of the IF-positive cells in the experimental fish

In the fish of control groups, no IF-positive cells were detected. IF-positive cells were first detected at 2nd day post-inoculation in the spleen and head kidney of the virus-inoculated fish (Table 1), and the number of
the positive cells remarkably increased at 6th day post-inoculation. In the liver and body kidney, the positive cells were first found at 6th day post-inoculation. The positive cell number was low in 10 days after inoculation, and no positive cells were detected in the liver at 14th day post-inoculation. The results obtained from the experiments with groups of both sizes revealed that the order of the organs on the prevalence of positive cell detection was generally spleen ≥ head kidney > body kidney > liver (Table 1). The number of cells detected in the group of small fish was higher than that in the group of large fish.

**Light microscopy**

The time course of histopathological changes was similar in the virus-inoculated fish from the two groups of each size. The histopathological changes in the spleen, head kidney, liver and body kidney of the inoculated fish were unremarkable at 2nd and 4th day post-inoculation. Eosinophilic degenerated cells were notably observed from 6th day post-inoculation in the spleen and head kidney of the inoculated fish, and basophilic enlarged cells were also frequently found in the organs. In the fish sampled at 8th day post-inoculation and the dead fish, the degenerated eosinophilic cells and debris were diffuse in the spleen and head kidney (Fig. 2). The histopathological changes were unremarkable in the liver and body kidney in the dead fish, but basophilic enlarged cells were also sometimes found in the small blood vessels of the liver and in the small blood vessels and the hematopoietic region of the body kidney. In the fish sampled at 14th day post-inoculation, which were survivors of these experimental infections, the eosinophilic cell debris in the spleen and head kidney was granulated, and the enlarged cells could not be seen.

Basophilic enlarged cells were found in the group of

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<th>Table 1. Detection of the IF-positive cell from the organs of the artificially RSIV-infected grouper Epinephelus malabaricus</th>
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<td><strong>Days post-inoculation</strong></td>
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Detection frequency was determined by the IF-positive cell number under a microscope with a ×10 objective lens as follows:
- : no positive cells in 1cm square, +: 1 cell in 1cm square to 3 cells per objective area, ++: 4 to 9 cells per objective area, +++: over 10 cells per objective area
virus-inoculated small fish more frequently than in the group of large fish. The eosinophilic degenerated cells were observed in the same degree in both groups of fish.

TEM
Multiplication of the RSIV was found in an enlarged cell in the spleen of a fish sampled at 4th day post-inoculation. The enlarged cell associated with the endothelial cell of small blood vessels and the number of enlarged cells with viral multiplication increased at 6th and 8th day post-inoculation. The enlarged cells with viral multiplication were characterized by well-developed cell organelles, such as endoplasmic reticulum and mitochondria, and a degenerated nucleus (Fig. 3). The multiplication occurred in a cytoplasm of the cell where the electron-staining density was comparatively low. Slightly enlarged cells with no viral particles, which could be the infected cells at the early viral multiplication stage, were sometimes observed in the fish at 6th day post-inoculation (Fig. 4). The viral particles could not be observed outside of the cells in the fish at 6th day post-inoculation. In the dead fish at 8th day post-inoculation, the viral particles were found not only in enlarged cells but also outside of the cells, especially in and around the disrupted enlarged cells and the small blood vessels in the spleen and head kidney (Fig. 5). In the degenerated cells, a small number of viral particles were fre-
Multiplication of RSIV in grouper

quently found in the dead and live fish sampled at 8th day post-inoculation (Fig. 6). The viral particles were also observed in the endothelial cells of small blood vessels in the spleen and head kidney, and some of the cells degenerated.

Immunohistochemistry

The basophilic enlarged cells in the head kidney of the two dead fish of the large fish group were stained with the monoclonal antibody M10, but the degenerated eosinophilic cells and endothelial cells of small blood vessels were not stained (Fig. 7).

Discussion

Our time course study on the RSIV-infected grouper, *E. malabaricus*, by the IF test with a specific monoclonal antibody showed that IF-positive cells formed first in the spleen and head kidney. Basophilic enlarged cells that were viral-productive were present in the blood vessels. These results suggest that the enlarged cells, which primarily formed in the spleen and head kidney of the infected grouper, migrate to other organs via blood vessels.

Viral particles were not found in red blood cells and lymphocytes of the diseased red sea bream, and the target cells of the virus were considered to be leukocytes of the red sea bream (Inouye *et al*., 1992; Kusuda *et al*., 1994). In this study, the basophilic enlarged cells may belong to a kind of leukocyte of the grouper, since the cells migrate via blood vessels and the characteristics of the cell are the same as those reported previously in the diseased red sea bream (Inouye *et al*., 1992; Kusuda *et al*., 1994).

The monoclonal antibody M10 used in this study recognizes a non-virion early protein, and it can detect the positive cell in the infected red sea bream earlier than the other monoclonal antibodies, which recognize virion proteins (Nakajima *et al*., unpublished data). Although it was not clear whether the viral particles were assembled in the IF-positive cells detected at 2nd day post-inoculation, the viral particles were already constructed in the enlarged cell within at least 4th day post-inoculation. Kusuda *et al*. (1994) reported that the growth curve of RSIV on BF-2 cells at 30°C reached a plateau at 3rd day post-inoculation. We assume that the progeny virus was able to infect the other target cells, which caused the number of IF-positive cells to increase dramatically and enlarged cells with no viral particles to appear at 6th day post-inoculation.

In the experimentally infected grouper, the notable pathological changes are the diffuse occurrence of degenerated cells in the spleen and head kidney (Sano *et al*., 2000). Similar histopathological changes were observed in the grouper infected with iridovirus in other countries, as reported previously (Chua *et al*., 1994; Danayadol *et al*., 1997). In immunohistochemistry, the monoclonal antibody M10 reacted with the enlarged cells and not with the eosinophilic degenerated cells in which a small number of the viral particles could be seen. Since M10 reacts with RSIV non-virion protein which is produced in early timing of virus multiplication process and thought to be necessary for virus propagation, the viral particles in the eosinophilic degenerated cells had been probably originated from virus-productive cells that were ingested into the cell.

Although the number of IF-positive cells in the group
of larger fish was smaller than that in the group of smaller fish, the prevalence of the cell degeneration was similar in both groups, which had the same cumulative mortality. Therefore, the appearance of diffusely spread eosinophilic degenerated cells in the spleen and head kidney should be a sign associated with the mortality of the infected grouper. The mechanism of occurrence of eosinophilic degenerated cell is not clear, but the ingested materials including viral particle may inhibit the physiological functions of the cell. Further research concerning the relationship between the appearance of the eosinophilic degenerated cell and fish mortality will be needed to define the pathogenicity of RSIV in the grouper, *E. malabaricus*.

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References


