ELECTRON MICROSCOPIC STUDY ON ENLARGED CELLS OF RED SEA BREAM, *Pagrus major* INFECTED BY THE RED SEA BREAM IRIDOVIRUS (RSIV, GENUS *Megalocytivirus*, FAMILY *Iridoviridae*)

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ABSTRACT

Most histopathologycal studies of the red sea bream iridovirus (RSIV) disease in red sea bream have been performed by studying enlarged cells as well as necrotized cells in the spleen and other organs. These enlarged cells have been named as inclusion body bearing cells (IBCs). However, few information is available about detail of ultrastructural features of IBCs produced in the target organs of RSIV-infected fish. In the present study, details of ultrastructural features of IBCs that were produced in the spleen tissue of naturally RSIV-infected red sea bream were investigated under electron microscope. Under electron microscope, RSIV-infected red sea bream had the presence of two types of IBCs: typical IBCs allowing virus assembly within viral assembly site (VAS), and atypical IBCs which degenerate organelles without virus assembly. Other infected-cells were observed as necrotized cells forming intracytoplasmic VAS with large numbers of virions, but without the formation of the distinct inclusion body. Morphogenesis steps on RSIV-infected red sea bream were observed as filamentous-filed virions, partially-filled virions and complete virions with 145-150 nm in size. These findings confirmed that RSIV-infected red sea bream were characterized by formation of typical and atypical IBCs as well as necrotized cells.

KEYWORDS: red seabream, RSIV, INCs, necrotized cells, VAS virion

INTRODUCTION

Iridovirids are well known as causative agents of several serious systemic diseases in a wide variety of fishes either wild, cultured or ornament, and have been identified to infect at least 19 fish species in the last decade (Piaskoski & Plumb, 1999; Hyatt *et al.*, 2000). The family *of Iridoviridae* consists of five genera: *Iridovirus*, *Chloriridovirus*, *Ranavirus*, *Megalocytivirus*, and *Lymphocystivirus* in which each genera consists of one or more virus species and strains (Chinchar *et al.*, 2005).

The genus of *Megalocytivirus* encompasses at least several viral isolates such as red sea bream iridovirus (RSIV), grouper sleepy disease iridovirus (GSDIV), sea bass iridovirus (SBIV), African lampeye iridovirus (ALIV), dwarf gourami iridovirus (DGIV), Taiwan grouper iridovirus (TGIV), rock bream iriovirus (RBIV), and infectious spleen and kidney necrosis virus (ISKNV) (Chinchar *et al.*, 2005; 2007). RSIV is known to cause an acute and highly contagious disease. Since 1990, outbreaks of this viral infection, which is named as the red sea bream iridoviral disease (RSIVD), have caused

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severe economic losses in Asian countries including Japan (Inouye *et al.*, 1992), Korea (Sohn *et al.*, 2000; Jung & Oh, 2000), Thailand (Sudthongkong *et al.*, 2002a), Taiwan (Chou *et al.*, 1998), and China (He *et al.*, 2001). This disease has caused mass mortality among cultured marine fishes including 18 species of *Perciformes*, one species of *Pleuronectiformes*, and one species of *Tetradontiformes* (Matsuoka *et al.*, 1996). A recent survey showed that more than 31 species of fishes are susceptible to this virus (Kawakami & Nakajima, 2002). Based on genetic variation and geographic distribution, RSIV isolate from Japan belongs to the genotype I along with other virus isolates from Korea, China, and Thailand. Viruses belonging to the genotype I are widely distributed among various fish species in many Asian countries (Song *et al.*, 2008).

Diseased fish become lethargic and experience severe anemia, petechia of the gill and enlargement of the spleen. RSIV is sensitive to chloroform and ether and unstable to heat but not affected by ultrasonic treatment or repeated freezing and thawing (Nakajima & Sorimachi, 1994). The genome of RSIV virus encods a major capsid protein (MCP) gene and 92 other putative open reading frames (ORFs) (Kurita *et al.*, 2002). The MCP gene accounts for about 45% of the total protein of the virus and is needed for the cleavage and packaging of viral DNA to form viable virions (Williams, 1996). This gene has been selected to analyze the phylogenetic relationships of iridoviruses (Go *et al.*, 2006; Imajoh *et al.*, 2007; Lu *et al.*, 2005) and has been confirmed to be the most suitable gene in detection and measurement of RSIV infection (Caipang *et al.*, 2003; Dang Thi *et al.*, 2007).

Diseased fish histopathologically displays enlarged cells containing an unique inclusion body (inclusion body-bearing cells: IBC) and necrosis of virus-infected cells in the spleen and hematopoietic tissue (Jung *et al.*, 1997; Sudthongkong *et al.*, 2002a,b). Similar to the RSIV-infected cells *in vivo*, RSIV can also replicate in several fish cell lines (*in vitro*) at suitable temperatures from 20 to 25°C, however, BF-2 (blue gill fry-2) and GF (grunt fin) cell lines were demonstrated to be optimal for RSIV infection and proliferation (Nakajima & Sorimachi, 1994; Imajoh *et al.*, 2004; Mahardika & Miyazaki, 2009). RSIV-infected cells in these cell lines display cellular enlargement as a definitive cytopathic effect. Under electron microscopic observation, RSIV-infected GF cells revealed the presence of two types of enlarged cells: IBCs, and cells allowing virus propagation within an intracytoplasmic virus assembly site (VAS) and having the fragmented or absent nucleus (Mahardika & Miyazaki, 2009). Thus, RSIV-infected cells *in vivo* and *in vitro* produced IBCs as well as necrotized cells, both of which are observed as enlarged cells under light microscope (Miyazaki, 2007). Ultrastructural features of IBCs derived from GSDIV-infected humpback grouper, *Cromileptes altivelis* has been described in detail (Mahardika *et al.*, 2004) as well as enlarged cells derived from RSIV-infected GF cells (Mahardika & Miyazaki, 2009). However, IBCs derived from RSIV-infected red sea bream has not been described in detail (Jung *et al.*, 1997; Sudthongkong *et al.*, 2002 a,b). In the present study, electron microscopic investigations were carried out to study the ultrastructure of IBCs of RSIV-infected spleen tissue of the red sea bream. The morphology and morphogenesis of RSIV were also observed within splenocyte cells.

MATERIAL AND METHOD

Diseased Red Sea Bream

Twenty diseased red sea breams (body weight: 30-50 g) that were in moribund state or had just died were collected from 2 net cage platforms in Ise bay of Mie Prefecture, Japan in September 21 and 22, 2006. Diseased fish were previously confirmed to be infected by RSIV based on several clinical signs such as severe anemia, petechia of the gill, and enlargement of the spleen, as well as positive result of PCR assay for RSIV. Although a few trichodina-like ciliates were observed on the gills in some examined fish, no bacteria were isolated from the spleen (Shinji Tanaka, personal communication).

PCR Analysis

The spleens of 10 diseased fish were processed for PCR analysis. DNA templates were extracted from about 10-20 mg of spleen tissue using ISOGEN (Wako Nippon Gene, Osaka, Japan) according to the manufacturer's protocol. In the present study, the primer sets (1-F and 1-R), which were derived from the DNA sequence of the 959-bp *Pst* I fragment of RSIV (Kurita *et al.*, 1998) were used. The PCR amplification was performed under conditions described previously by Sudthongkong *et al.*

(2002b). Using this PCR test, amplification products of about 570 bp were generated.

Histopathological Examination

Pieces of the visceral organs (spleen, kidney, liver, and digestive tracts) were fixed in 10% phosphate-buffered formalin for histopathological examination. The fixed tissues were prepared according to Jung *et al.* (1997).

Electron Microscopic Examination

Tissue samples of the diseased fish were fixed in 70% Karnovsky's solution, post-fixed in 1% OsO_4 and processed for examination under electron microscope according to the method of Sudthongkong *et al.* (2002a).

RESULT AND DISCUSSION

Diseased Fish

The red sea bream mortality occurred during the summer season. The cumulative mortality reached about 50% to 70% in 2 net cage platforms. Individual diseased fish usually showed petechia of the gill and enlargement of the spleen. These findings were similar to those reported by Dr. Shinji Tanaka (personal communication) as well as the other scientists (Inouye *et al.*, 1992; Nakajima *et al.*, 1994; Jung *et al.*, 1997).

PCR Analysis

A number of primer sets specific for RSIV have been designed based on the ATPase gene, DNA polymerase gene, ribonucleotide reductase small subunit and *Pst I* restriction fragments of virus genomic DNA (Miyata *et al.*, 1997; Kurita *et al.*, 1998; Oshima *et al.*, 1998). In this study, one RSIV specific primer set, 1-F and 1-R was used in the PCR designed to identify suspected viral nucleic acid in the tissues of infected fish. A PCR product of about 570 bp was amplified using the 1-F and 1-R primer set with DNA template obtained from affected tissues. Alternatively, no product was found when using the tissues of healthy fish as a template (negative control). These results indicated that the viral agent in the cultured red sea bream was related to the RSIV (Figure 1).

Histopathological Examination

Of the 20 diseased fish, all spleens contained many enlarged cells as well as necrotized cells (Figure 2A, B). The spleens also contained focal or diffuse hemorrhage and melano-macrophage center (Figure 2C). In the
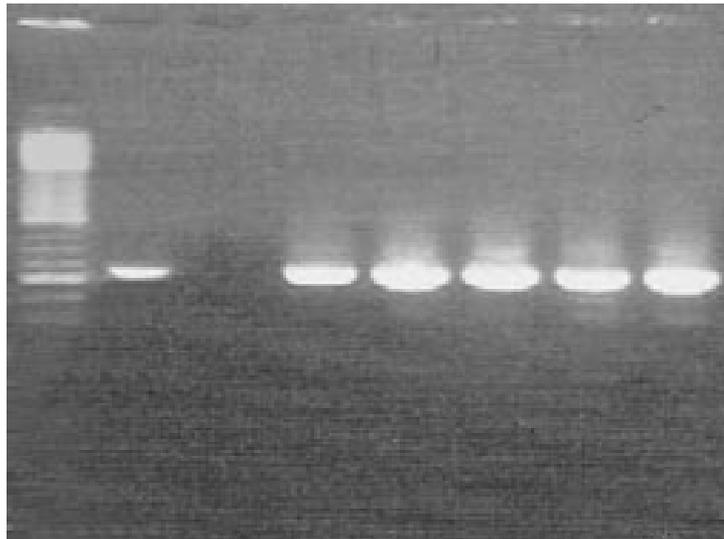

Figure 1. Putative PCR amplicons derived from DNA extract of the spleens of naturally moribund or dead fish (lane: 1 to 5). These results indicated that red sea bream were infected by RSIV (red sea bream iridovirus). M: marker, P: positive control, N: negative control
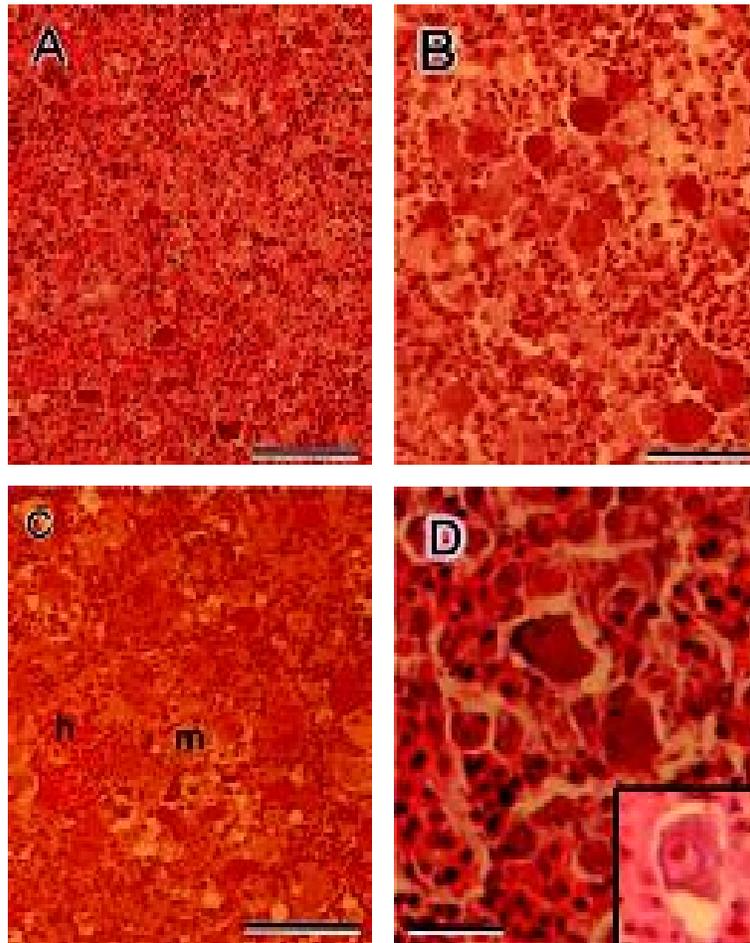
Figure 2. (A) Spleen of the RSIV-infected red sea bream shows massive formation of enlarged cells, necrotized plenocytes and diffuse hemorrhage (H&E; scale bar= 50 mm). (B) High magnification of enlarged cell observed in the spleen tissue (H&E; scale bar= 35 mm). (C) Spleen of the diseased fish contains a mass of melanomacrophage center (m) and focal hemorrhage (f) within formation of enlarged cells and necrotized splenocytes (H&E; scale bar= 50 mm). (D) High power view of typical IBC (inclusion body bearing cell), nucleus of host cell is marginally compressed (black color) (H&E; scale bar= 20 mm). Insert, atypical IBC forms nucleus-like structure in the central of cell, while nucleus of the host cell was pyknotic and marginally compressed.

kidney, many enlarged cells appeared in the hematopoietic tissues and glomerular capillary. Some enlarged cells infiltrated into the liver and tunica propria mucosae and submucosae of the digestive tracts. These enlarged cells contained basophilic inclusion body within the cytoplasm, whose nucleus located at the periphery (Figure 2D). These enlarged cells were named as inclusion body bearing cells (IBCs)

(Jung *et al.*, 1997; Sudthongkong *et al.*, 2002 a,b; Mahardika *et al.*, 2004, 2008; Miyazaki, 2007).

Electron Microscopy Examination

Under electron microscope, typical IBCs had an intracytoplamic inclusion body, which was delimited from the host-cell cytoplasm by

a thin membranous boundary. Early stage of IBCs formed dark matrix of a small inclusion body with few virions. Host cell nucleus became marginally compressed (Figure 3A). Mature IBCs had an enlarged inclusion body containing a developed virus assembly site (VAS), abundant mitochondria, ribosome, smooth endoplasmic reticulum (sER), rough granules and/or smooth granules with high electron density. The host cell cytoplasm was marginally located and contained mitochondria, ribosome, sER and nucleus with irregular shapes (Figure 3B, 3C). Markedly enlarged IBCs had a markedly enlarged inclusion body containing a developed VAS, many mitochondria, sER, ribosome, and masses of rough granules (Figure 3D). Another markedly enlarged IBCs, showed a marginally compressed nucleus and cytoplasm surrounding an enlarged inclusion body. Their inclusion bodies had a centrally located VAS, abun-
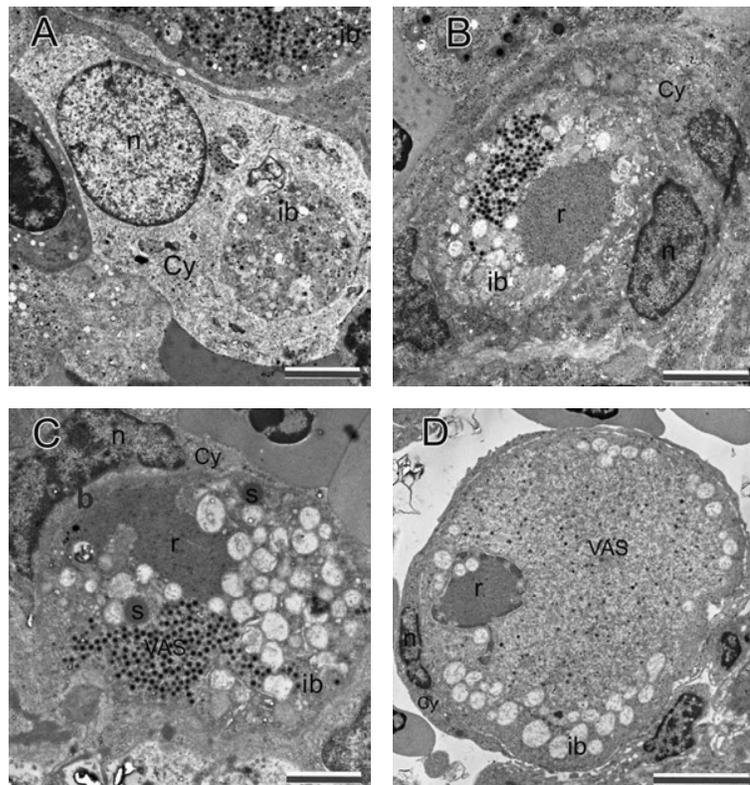

Figure 3. Electron micrograph of spleen tissue of the diseased fish. (A) Early stage of IBC forms dark matrix of a small inclusion body with few virions. Host cell nucleus became marginally compressed (scale bar= 2,000 nm). (B) Small mature IBC has an inclusion body containing a developed virus assembly site (VAS). VAS contains mass of rough granules containing viral DNA and assembly of virus particles (scale bar= 2500 nm). (C) Small mature IBC has an intracytoplasmic inclusion body which delimited from the host-cell cytoplasm by a thin membranous boundary. It inclusion body contains developed VAS with virions propagation, abundant mitochondria, ribosome, sER, and rough and smooth granules. Host cell nucleus is marginally compressed with irregular shape (scale bar= 2,500 nm). (D) Mature IBC has an enlarged inclusion body containing rough granules that resemble a nucleus but had no surrounding double membranes, and developed VAS with propagated virions (scale bar= 3,600 nm). b: boundary membrane; Cy: cytoplasm of host cell; ib: inclusion body; n: nucleus of host cell; r: rough granules; s:smooth granules; RBC: red blood cell; VAS: virus assembly site

dant ribosomes, mitochondria, sER and masses of rough and smooth granules (Figure 4A, 4B). The highly ballooning cells had an inclusion body with a clear appearance within the surrounding thinned cytoplasm. Their inclusion body contained degenerated and fragmented organelles and a decreased number of virions in the VAS (Figure 4C). Masses of rough and smooth granulars were fragmented among VAS and budding virions on the surface of the host cell cytoplasm. The VAS of mature IBCs had an electron-lucent matrix with abundant fine gran-

ules, microfilament, and multiple virus particles. Morphology and morphogenesis of the virus were detected. In the VAS, filamentous-filled virions, partially-filled virions, and hexagonal fully-filled virions (complete virions) were observed among microfilaments and fine granules (Figure 4D, 5A). Filamentous-filled virions had filamentous nucleoprotein core with capsid without definite capsomers. Partially-filled virions were a little hexagonally deformed and comprised the partially package of an electron-dense core within the capsid without defi-
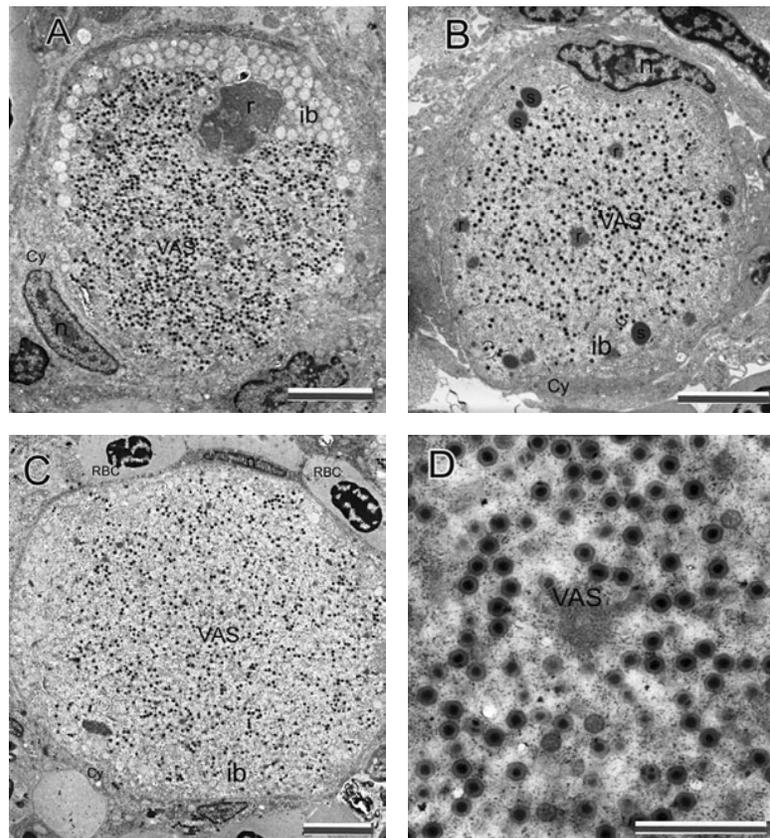

Figure 4. Electron micrograph of spleen tissue of the diseased fish. (A) Mature IBC with markedly enlarged inclusion body contains VAS with fully virions, mitochondria, ribosome and rough granules beside VAS (scale bar= 3,700 nm). (B) Enlarged IBC with an inclusion body contains VAS with many virions at all morphogenesis steps. Rough and smooth granules are fragmented within VAS (scale bar= 3,500 nm). (C) The highly ballooning cell has an inclusion body with a clear appearance within the surrounding thinned cytoplasm. It inclusion body contains degenerated and fragmented organelles and a decreased number of virions within VAS (scale bar= 3,500 nm). (D) High-power view of viral assembly site (VAS). It contains fine granules and microfilaments with assembly of RSIV virions (scale bar= 1,200 nm). Abbreviations as in Figure 3
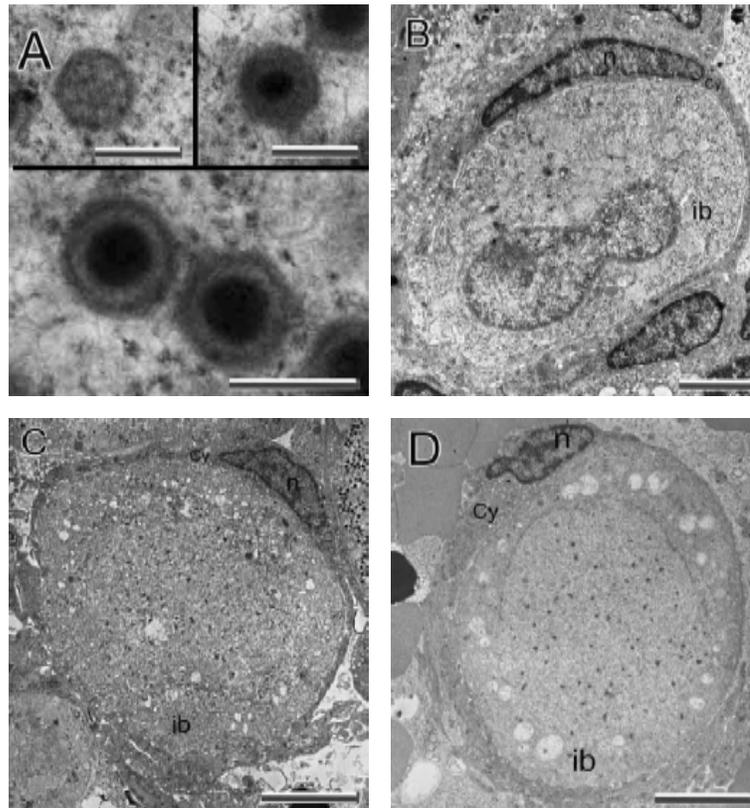
Figure 5. Electron micrograph of spleen tissue of the diseased fish. (A) Detailed features of virus particles in all stages of morphogenesis within the VAS of IBC. There are filamentous-filled virions, partially-filled virions, and hexagonal fully-filled virions (complete virions) with the size of 145 to 150 nm (scale bar= 150 nm). (B) Atypical IBC, the host cell cytoplasm surrounded an inclusion body with the electron-lucent matrix containing abundant granules, mitochondria, rER, and a swollen nucleus-like structure (scale bar= 2,500 nm). (C) Atypical IBC with inclusion body contains fragmented of masses of granules without nucleus-like structure (scale bar= 3,500 nm). (D) Atypical IBC has inclusion body without the formation of VAS and it degenerates without virion assembly (scale bar= 3,600 nm). Abbreviations as in Figure 3

nite capsomers. Complete virions were 145–150 nm in size, comprising, in turn, a complete package of an electron-dense core, and a capsid with capsomers.

Typical IBCs of the red sea bream were mostly the same as those reported previously by other scientists (Jung *et al.*, 1997; Sudthongkong *et al.*, 2002 a,b; Mahardika & Miyazaki, 2009; Miyazaki, 2007) as well as typical IBCs found in the other members of megalocytivirus (Mahardika *et al.*, 2004, 2009; Miyazaki, 2007). Other viruses, such as Euro-

pean catfish virus (ECV; *Ranavirus*) and lymphocystis disease virus (LCDV; *Lymphocystivirus*) in the family of *Iridoviridae* have been known to form intracytoplasmic inclusion bodies (Hyatt *et al.*, 2000; Miyazaki, 2007). However, the features of these inclusion bodies are completely different from those of the inclusion bodies that are grown in the IBCs of RSIV-infected cells. The early stage of IBCs that had already formed a small inclusion body were the same as those that were observed in RSIV (Jung *et al.*, 1997; Miyazaki, 2007). The feature of inclusion bodies at the

early stage of IBCs resembled to that of mature IBCs except for few virions or a small number of virions being assembled.

In IBCs, the inclusion bodies are composed of virions, rER, sER, ribosomes, mitochondria and the granular matrix that contains DNA. Chinchar *et al.* (2005) reported that synthesis of viral DNA viruses of the family *Iridoviridae* was known to occur within the nucleus and the cytoplasm. Viral DNA replication commences within the nucleus and then newly-synthesized viral DNA molecules are subsequently transported to the cytoplasm where they serve as templates for the formation of concatameric DNA, that is, the second stage DNA synthesis. The morphogenetic stages of RSIV resembled those in other members of megalocytivirus (Chao *et al.*, 2004; Mahardika *et al.*, 2004; Miyazaki, 2007) as well as ranavirus-infected cells (Hyatt *et al.*, 2000; Chinchar, 2002; Chinchar *et al.*, 2005).

Atypical IBCs were also observed, which were different from typical IBCs. The host cell cytoplasm surrounded an inclusion body with the electron-lucent matrix containing abundant granules, mitochondria, rER, and a swollen nucleus-like structure (Figure 5B). Some inclu-

sion bodies of atypical IBCs contained fragmented of masses of granules without nucleus-like structure (Figure 5C, 5D). The abnormality of the inclusion body was also substantiated by the formation of none of VAS and it degenerated without virion assembly. This indicated that RSIV infected cells and induced them to produce an IBC. However, some RSIV virus failed to replicate among inclusion bodies and then the IBC was degenerated and fragmented without virus assembly. Similar abnormal IBCs were also observed in GSDIV-infected humpback grouper which was assumed that unknown antiviral factors were performing antiviral activity within the systemic defense system and inhibiting virus assembly in the spleen before the fish became moribund (Mahardika *et al.*, 2004).

Similar to IBCs, necrotized cells were also observed. This cell formed intracytoplasmic VAS without the formation of the distinct inclusion body. In the VAS, large numbers of virions were assembled (Figure 6A, 6B). Some of these cells contained pyknotic nuclei, and other necrotized cells had fragmented nuclei and the nuclear components were assimilated with the cytoplasm, resulting in the disappearance of the nuclei. These cells developed intracy-
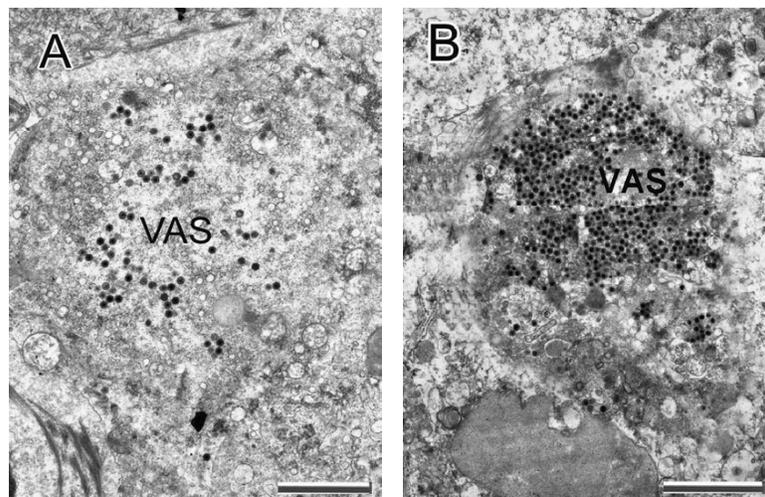

Figure 6. Electron micrograph of the spleen tissue of the diseased fish. (A) A splenocyte infected with RSIV is necrotized with propagation of GSDIV in the cytoplasm. This type of cell have just formed intracytoplasmic VAS, in which some virions are being assembled, without the formation of distinct inclusion body (scale bar= 1,500 nm). (A) Necrotized splenocyte contains large number of virus particles within intracytoplasmic VAS. The nucleus has disappeared (scale bar= 2,000 nm). Abbreviations as in Figure 3

toplasmic VASs, within which many virions propagated. This cell was also observed in megalocytivirus-infected red sea bream and grouper fishes (Mahardika *et al.*, 2004; 2009; Miyazaki, 2007; Mahardika & Miyazaki, 2009). These cells are also one of the characteristics of megalocytivirus infection.

The occurrence of RSIV infection in red sea bream appeared to be correlated with temperature fluctuation. The water temperature had risen when the epizootic mortalities occurred. The RSIV outbreaks in Japan occurred in summer temperatures. However, in Japan, RSIV led to the losses of up to 20%–60% in fingerling and market-sized red sea bream (Nakajima *et al.,* 1998). The spleen and kidney were the most affected organs in the infected fish. Therefore, histopathological observations were consistent with the findings of anaemia and splenomegaly in RSIV-infected red sea bream. Development of typical and atypical IBCs as well as necrotized cells in the spleen and kidney of affected fish characterizes the RSIVD. Morphogenesis of RSIV virus particles were detected in the viral assembly site in both of typical IBCs and necrotic cells. Hexagonal mature or complete virions found in RSIV was 145-150 nm in diameter, and thus smaller than TGIV (Chou *et al.,* 1998) and GSDIV from Indonesia (Mahardika *et al.,* 2004). They were similar to RSIV found in cultured GF cells (Mahardika & Miyazaki, 2009).

In summary, utrastructure investigation is the main step to define the characterization of the RSIV-infected red sea bream. RSIV-infected red sea bream induces typical and atypical IBCs as well as necrotized cells. Typical IBCs and necrotized cells contain virus particles with all morphogenesis steps as immature virions (filamentous filled virion and partially filled virion) and mature virions. While atypical IBCs are degenerated without virus assembly.

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