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Ultrastructures of Lymphocystis Cells in Japanese Flounder *Paralichthys olivaceus*

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Abstract

Histological signs and ultrastructures were studied on young and grown lymphocystis cells (LCCs) occurring in farmed Japanese flounder *Paralichthys olivaceus*. Young LCCs had a basophilic cytoplasm containing highly basophilic reticulations of inclusion bodies and a hypertrophied nucleus including prominent nucleoli, and were encapsulated with a thick hyaline wall. The cytoplasm extended many protrusions into the wall and was abundant in viral assembly sites (AS), rough endoplasmic reticula (rER), ribosomes, smooth endoplasmic reticula and mitochondria. AS consisted of inclusion zones on which viral particles began to be assembled and the surrounding matrix containing various maturing stages of virions. Grown LCCs had an eosinophilic cytoplasm displaying marginally located inclusion bodies with a decreased number and size. The cytoplasm displayed enormously large numbers of virions (250 to 300 nm in edge to edge diameter) showing crystalline array, and degenerated rER and mitochondria within the inner regions. The marginal areas of the cytoplasm contained a decreased size of AS, degenerated organelles and vacuoles. The nuclear membranes and cytoplasmic protrusions were degenerated, indicating that these cells were mature or old. Some fibroblasts formed inclusion bodies indicating LCCs derived from fibroblasts.

Key Words: Lymphocystis cells, *Paralichthys olivaceus*, inclusion bodies, viral assembly sites, inclusion zones

Introduction

Lymphocystis disease is caused by *Lymphocystivirus* belonging to the Family *Iridoviridae*[1]. This disease is a well-known virus disease that occurred in not only cultured fishes but also wild fishes in all over the world: i.e., North America[2-5], Europe[6,7], the Middle and near East[8-10], Australia[11] and Japan[12,13]. Lymphocystis disease is characterized by the formation of lymphocystis cells that are derived from lymphocystivirus-infected cells and show markedly hypertrophic features and intracytoplasmic inclusions. Although fine structures of lymphocystis virus within lymphocystis cells had been stated in many fish species[9,10,13-18], our understanding of ultrastructures of lymphocystis cells is less advanced[8-10,14]. Lymphocystis cells have been recognized to be a typically hypertrophied cell. As well as lymphocystivirus, other iridoviruses-infected cells were claimed to be hypertrophied or enlargement- i.e., RSIV (madai iridovirus)[19], WSIV (white sturgeon iridovirus)[20], gourami iridovirus[14] etc. In order to compare with features of hypertrophied cells due to other

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iridoviruses, accumulation of ultrastructural findings of lymphocystis cells is worthwhile. In the present study, we revealed ultrastructures of the different stages (early stage, young and mature) of lymphocystis cells formed in Japanese flounder *Paralichthys olivaceus*.

Materials and Methods

A total of 10 cultured Japanese flounders (around 100 g BW) with lymphocystis disease were collected from ponds on land in winter 2000 and 2002. Pieces of nodules consisted of lymphocystis cells (LCCs) were removed from the body and fins, and fixed in Bouin's fluid for light microscopic observation. The fixed tissues were prepared according to standard techniques and stained with hematoxylin & eosin (H&E), Azan and Feulgen reaction for DNA. Other pieces were fixed in 70% Karnovsky's solution, postfixed in 1% OsO4 and processed for electron microscopy.

Results

External and histopathological signs

All of diseased flounders displayed many nodular lesions consisted of large numbers of LCCs in the skin of all over the body including eyes, noses, jaws and fins. Some diseased fish displayed flat nodular lesions extended over the trunk (Fig. 1A). The other fish had large massive lesions including blood vessels in fins (Fig. 1B). The formation of these lesions was not lethal to diseased fish while some of them died of feeding disturbance due to the formation of nodular lesions on the jaws.

In a histological examination of nodular lesions, many LCCs with various sizes had grown within the dermis. LCCs were markedly hypertrophied cells bounded with a hyaline wall (Fig. 2A). Small and young LCCs (50-250 μm) had a thin wall and a slightly basophilic cytoplasm including a small number of highly basophilic inclusion bodies with a reticulate structure around a hypertrophied nucleus possessing a prominent nucleolus and highly basophilic chromatin (Fig. 2B). Well-grown LCCs (>about 300 μm) formed a thick hyaline wall and had a cytoplasm showing a granular appearance (Fig. 2C). Their inclusion bodies were increased in number and had a complex reticulation that consisted of a highly basophilic reticulation and the surrounding basophilic substance in the marginal areas of the cytoplasm (Fig. 2D). Feulgen reaction to the inclusion bodies revealed many

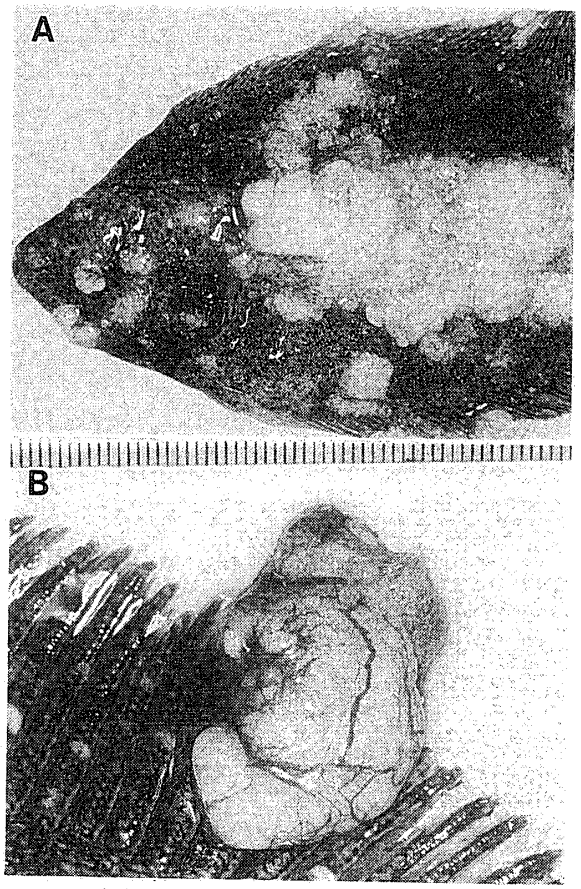

Fig. 1 (A) Flounder showing flat nodular lesions consisting of large numbers of lymphocystis cells (LCCs) on the trunk. (B) Flounder showing a big nodular lesion in the dorsal fin.

thin reticulations of Feulgen-positive matter were embedded within the inclusion body (Fig. 2E). These findings indicated that the inclusion body consisted of DNA reticulations and the surrounding basophilic matrix. An enlarged nucleus contained an increased amount of highly basophilic chromatin forming a complex reticulation along with partial coagulation, and increased fine granular chromatin. Degenerating LCCs had an eosinophilic granular cytoplasm with marginally-located vacuoles and fragmented inclusion bodies (Fig. 2F). A swollen nucleus possessed destructive nucleoli and a markedly decreased amount of the highly basophilic chromatin. Some of LCCs had been ruptured and infiltrated by many host cells within a remained hyaline wall (Fig. 2A). Beside them, the early stage of LCCs ($<30\,\mu m$) appeared, that had an basophilic cytoplasm with a few of small basophilic inclusion bodies and an enlarged nucleus with increased basophilic chromatin (Fig. 2D).

Electron microscopy of LCCs

In electron microscopy of LCCs, all of LCCs were encapsulated by a thick hyaline wall that consisted of an amorphous matter containing large numbers of fine granules and was penetrated by many cytoplasmic protrusions. Young LCCs already had a hyaline wall which was penetrated by the membrane-bounded cytoplasmic protrusions forming microvillus-like, dendrite-formed or mesh-work structures (Fig. 3A). The cytoplasm was abundant in dark matrix, rough endoplasmic reticula (rER), ribosomes, smooth endoplasmic reticula (sER), mitochondria and viral assembly sites (AS). All of rER were isolated each other. Although AS was not sharply delimited from cytoplasmic organelles, AS consisted of many inclusion zones of aggregated granules with high-electron density and the surrounding low electron-dense matrix zones containing many virions and a paucity of organelles (Fig. 3B). Within AS, various developmental stages of virus particles were observed (Fig. 3 C, D). On the inclusion zones, hemi-hexagonal or tri-angler capsids were formed to encapsulate the granular material, indicating it was the early stage of virion assembly. In the matrix zone around the inclusion zone, there were a large number of virions possessing a hexagonal outline of a capsid with a spiked surface and a core of either granules or a low electron-dense material. A small number of completed virions with an electron-dense capsid and an electron-dense core were mainly present at the peripheral areas of the matrix zone. A nucleus had double membranes with a well distance and was packed with abundant fine granular chromatin and condensed chromatin with high electron-density, and enlarged nucleoli. There was no virion in the nucleus.

Well-grown LCCs had an electron-lucent cytoplasmic matrix. The inner regions of LCC were replaced by large numbers of virions. Most virions were completed ones (250 to 300 nm in edge-to edge diameter) and showed crystalline array (Fig. 4A, B). Thus, AS and cytoplasmic organelles were mostly destroyed and mixed with virions. All of remained rER lost ribosomes from the surface and agglomerated each other. Mitochondria disappeared within these regions. In the marginal areas of the cytoplasm, although AS remained, the matrix zones were loosened and the inclusion zones were decreased in size or fragmented while virion assembly slightly continued on them. The matrix zone held many mature virions (Fig. 4 C). Around AS, cytoplasmic organelles were markedly degenerated. Most of remained rER lost ribosomes from the surface and were deformed, and some of them agglomerated. Tubular or cylindrical structures were not observed. The nucleus had the wrinkled membranes which were partially destroyed. Along the inner membrane, chromatin formed many ring structures (Fig. 4A). Within the hyaline wall, cytoplasmic protrusions were more extended and formed the complicated structures. The boundary membrane of

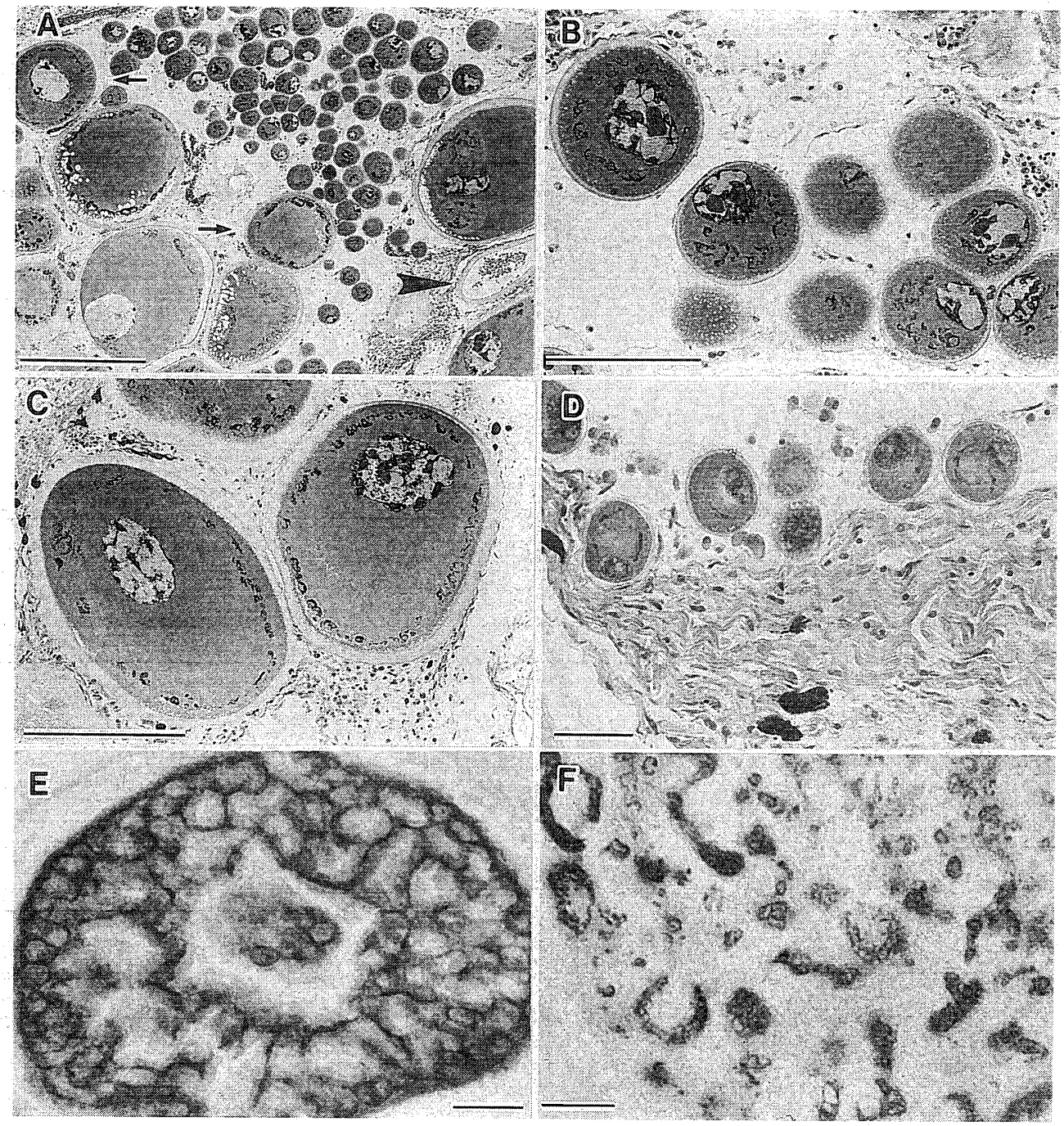

Fig. 2 (A) Many small lymphocystis cells (LCCs) and young LCCs (arrows) appeared beside ruptured LCCs whose hyaline wall only remained (arrow head) in the dermis of the trunk. Well grown LCCs were vacuolated. H & E, Scale bar=30 μm. (B) Detail of small LCCs that had a thin hyaline wall, inclusion bodies and an enlarged nucleus with a prominent nucleolus and much chromatin. H & E, Scale bar=10 μm. (C) Well grown LCCs. H & E, Scale bar=20 μm. (D) Early stage of LCCs. Some inclusion bodies developed in the hypertrophied cytoplasm. H & E, Scale bar=3 μm. (E) High-power view of an inclusion body. It formed a basophilic reticulation. H & E, Scale bar=20 μm. (E) High-power view of Feulgen-positive inclusion zones that were embedded within an inclusion body. Feulgen reaction. Scale bar=10 μm.

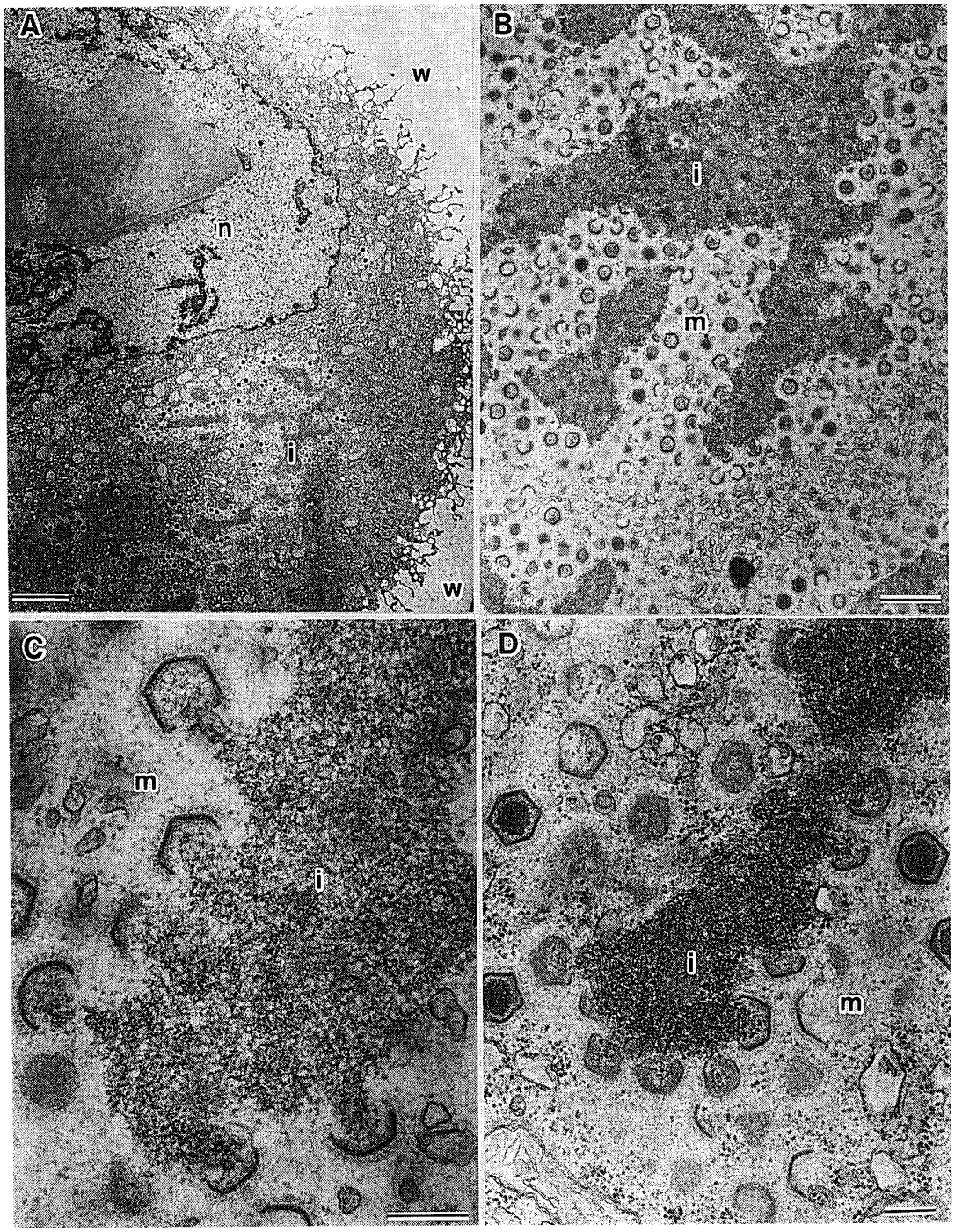

Fig. 3 Electron micrographs of young lymphocystis cells (LCCs). (A) Low magnification view. The cell had a hyaline wall and extended cytoplasmic protuberances into the wall. The cytoplasm was abundant in viral assembly sites containing inclusion zone, rER, ribosomes and mitochondria. The nucleus contained an enlarged nucleolus and was abundant in euchromatine and highly dense chromatin. Scale bar=3000 nm (B) Enlarged view of viral assembly sites (AS). AS consisted of inclusion zones and the matrix zones containing many viral particles. Scale bar=1000 nm. (C) High power view of AS. On the inclusion zone, virus particles showed the various assembly stages. Scale bar=300 nm. (D) High power view of AS. Virus particles were assembled on the inclusion zone and completed virions were located at the marginal matrix zone. Scale bar=300 nm. i: inclusion zone. m: matrix zone. n: nucleus. w: hyaline wall.

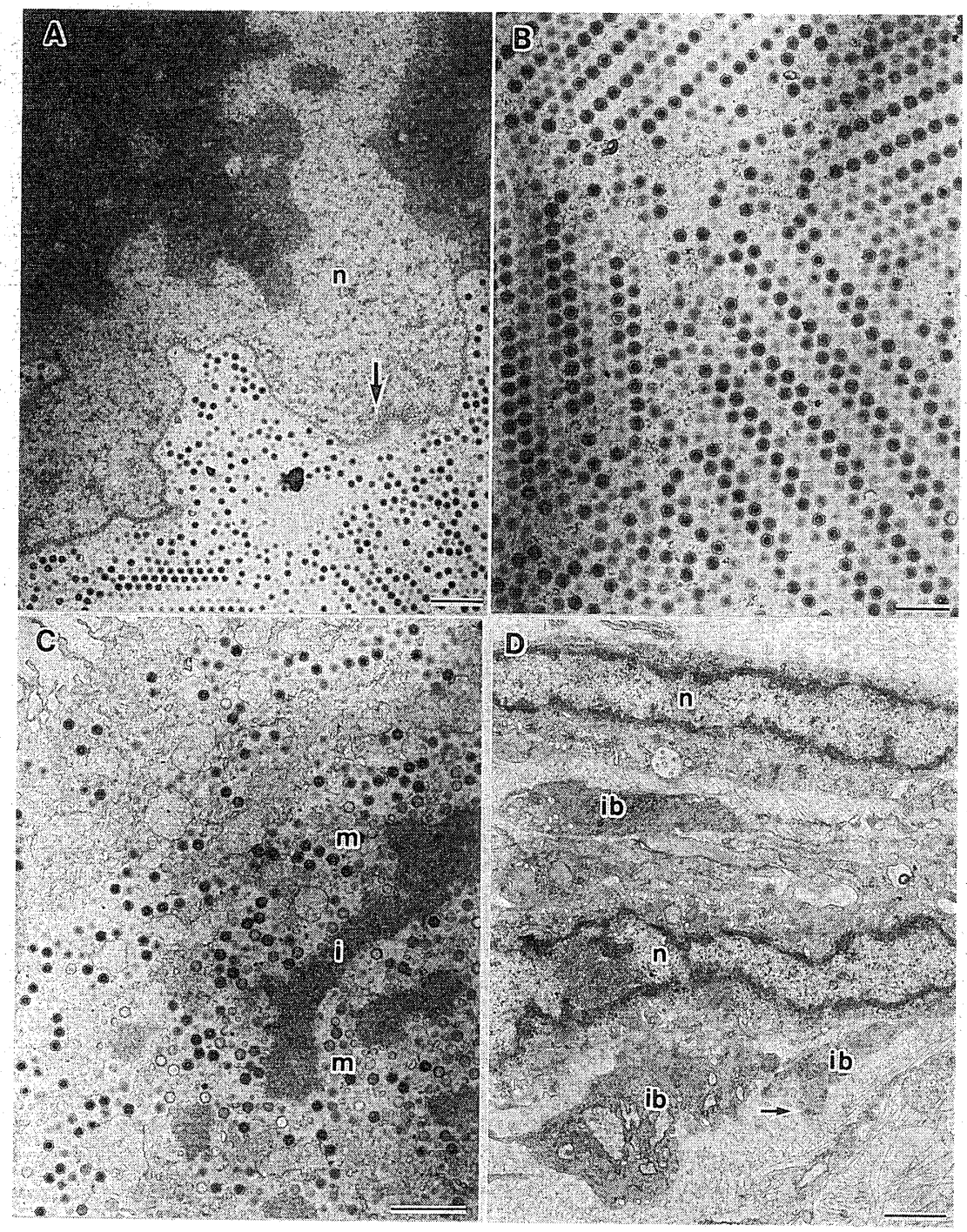

Fig. 4 (A-C) Electron micrographs of mature lymphocystis cells (LCCs). (A) Central region of LCC. The nuclear membranes damaged and along the inner membrane, chromatin formed many ring structures (arrow points). The cytoplasm of this region was replaced by large numbers of completed virions. Scale bar=2000nm. (B) In the inner region of cytoplasm, completed virions formed crystalline array within destroyed viral assembly sites (AS). Scale bar=1000 nm. (C) Marginal area of LCC. AS was fragmented and destroyed. Most organelles were degenerated. Scale bar=2000 nm. (D) Electron micrograph of fibroblasts. One cell showed a small inclusion body that a virion (arrow points) had been assembled. Scale bar=1000 nm. i: inclusion zone. ib: inclusion body. m: matrix zone. n: nucleus.

cytoplasmic protrusions was partially destroyed resulting in exudation of a cytoplasmic matrix into the wall. There was no virion present within the protrusions and the wall.

The early stage of LCCs were revealed as fibroblasts forming AS in which a few virus particles had been assembled, although they were not rounded and did not form a hyaline wall around the cell (Fig. 4D).

Discussion

As all reports had stated, LCCs had intracytoplasmic inclusion bodies. Feulgen reaction revealed that Feulgen-positive reticulations were embedded within inclusion bodies. Electron microscopy of young LCCs revealed that AS consisted of inclusion zones of aggregated granules and the surrounding matrix zone with a paucity of organelles. Because virus particles were assembled on the inclusion zones, the inclusion zones were made up of viral DNA and must be the Feulgen-positive reticulations in histological observation. In electron microscopy, various developmental stages of virions could be traced while virions moved from the inclusion zones to the peripheral areas of the matrix zone within AS. The viral DNA appeared to be synthesized in the inclusion zones, although the viral DNA templates might be propagated within a hypertrophied nucleus and transferred into the cytoplasm. Lipids were revealed to be present within virions because lymphocystivirus were sensitive to ether[21]. These findings indicated that the AS matrix contained proteins and lipids to produce viral particles. Because the AS matrix contained a paucity of rER, the matrix proteins were synthesized by the surrounding cytoplasmic rER and ribosomes. The AS feature was the same as that of LCCs of snakeskin gourami, *Trichogaster pectoralis*[9,13]. Based on electron microscopic findings of young LCCs, the entire AS was observed as a basophilic inclusion body in a light microscope. Because the cytoplasm was abundant in organelles, the cytoplasm showed basophilic appearance in a light microscope. Abundant cytoplasmic organelles contributed to not only the propagation of virions but also the growth of LCCs along with a hypertrophied nucleus that was packed with chromatin and prominent nucleoli.

In well-grown LCCs that histologically had an eosinophilic cytoplasm and marginally located inclusion bodies, the cytoplasm was widely replaced by large numbers of virions due to AS destruction, and markedly degenerated cytoplasmic organelles as rER and mitochondria. These features resembled to those of grown LCCs in *Sparus aurata*[8], scalare *Pterophyllum scalarae*[10] and pike perch (=walleye) *Stizostedion vitreum*[4,14]. Because the grown LCCs had damaged nuclear membranes, these LCCs were in a mature or old stage that synthetic activities were decreased or ceased.

As all reports had stated, LCCs had a thick capsule or a hyaline wall. Such a wall was inferred to be thickened with LCC produced materials, and to protect LCC from the attack of host macrophages and fibrocytes as the defense reaction and from burst due to enormously increased cellular contents until the time when lymphocystivirus propagation has been completed. Cytoplasmic protrusions penetrated into the wall so that LCCs make the nutrients acquisition more efficient through the thick wall. The cytoplasmic protrusions of flounder LCCs and walleye LCCs[4] were much more extended than those of LCCs of *S. aurata*[8], snakeskin gourami[9] and kurosoi *Sebastes schlegeli*[13]. Because the cytoplasmic protrusions had a boundary membrane, the wall was not a thickened plasma membrane and appeared to consist of the excreted substance by abundant organelles in the bottom of the protrusions. Cultured BF-2 cells that were infected with lymphocystivirus also produced a thick wall[22,23]. Thus, the production of a thick hyaline wall is a nature of lymphocystivirus-infected cells. Because the wall did not allow virion passage through, virions

appeared to be released due to burst of grown LCCs in the tissues. Because LCCs were known to be naturally separated from fish, virions appeared to be released in the water after destruction of the hyaline wall.

Lymphocystivirus has been cultured, and cultured BF-2 cells that were infected with bluegill lymphocystivirus also formed AS[16], however, the AS feature was different from that of LCCs occurred within fish tissues. On the contrary, FHM cells that were infected with a ranavirus of ECV (European catfish iridovirus)[24] displayed similar AS to that of LCCs.

Electron microscopic findings of LCCs indicated that LCCs were enormously hypertrophied cells and the hypertrophy contributed to propagation of immensely large numbers of lymphocystivirus. Thus, LCCs were the factory to produce virions. As compared with other iridoviruses-infected cells, ranavirus-infected FHM cells[24] displayed the similar AS although the infected cells were slightly hypertrophied. As well as RSIV-infected cells of red sea bream *Pagrus major*[19], iridovirus-like agent-infected cells of gouramies *Trichogaster trichopterus* and *T. leeri*, and swordtail *Xiphophorus hellerii*[10] were also claimed to be hypertrophic. However, electron microscopic examinations of RSIV-infected cells revealed the formation of a unique inclusion body that had been sharply delimited within the host cell cytoplasm[25]. The feature of the early stage of inclusion body was different between LCCs and RSIV-infected cells[25]. The growth of the RSIV inclusion body caused the enlargement of host cells while the host cell cytoplasm and nucleus were marginally compressed and atrophied[25]. The formation of the same inclusion body was observed in cells infected with DGIV (dwarf gourami iridovirus) and ALIV (African lampeye iridovirus) from Southeast Asian countries[26]. RSIV, DGIV and ALIV were revealed to be genetically the same and have been classified in a new genus *Tropivirus*[27] in the Family *Iridoviridae*. Because the growth of the unique inclusion body caused the enlargement of host cells, the host cell nucleus was hypertrophied so that the hypertrophied nucleus served for the growth of the inclusion body. These *Tropivirus*-infected cells were recognized to be enlargement due to the growth of the unique intracytoplasmic inclusion body.

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ヒラメ *Paralichthys olivaceus* のリンホシスチス細胞の微細構造

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養殖ヒラメに発生したリンホシスチス細胞（LCC）の微細構造を観察した。若いLCCは既に厚い硝子状被膜を持って肥大しており，細胞質にはウイルス合成の場（AS），粗面小胞体，リボゾーム，ミトコンドリアがよく発達し，細胞質の樹状の突起が被膜内に伸張していた。ASは顆粒状のinclusion zoneと基質部から構成され，inclusion zone表面でウイルス粒子が合成されていた。大きく肥大したLCCの核周囲の細胞質内ではASが崩壊し，ウイルス粒子（250-300nm）は結晶配列を示していた。細胞辺縁部では，多数のウイルス粒子を含むASは明瞭であるが，微小器官の多くは変性していた。