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***In vitro* comparative ultrastructural observations on fish rhabdoviruses (HRV, IHNV, PFR, EVA and EVEX)**

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Abstract

Ultrastructural observation on fish rhabdoviruses, hirame rhabdovirus (HRV): *Rhabdovirus olivaceus*, infectious haematopoietic necrosis virus (IHNV), pike fry rhabdovirus (PFR), eel virus of America (EVA) and eel virus of Europe (EVEX) on RTG-2 cells was studied at 12 hr post-infection. Virus particles were found budding on the cell surfaces and were never observed in the vacuoles. Club-like or cord-like structures were easily observed on the cell surface of HRV infected cells. Appearances of these structures sometimes varied from club-like to cord-like structures, but all of them showed high electron density and included microfilaments. Most of them contained many viral particles on the surface. However, it was difficult to find such club- or cord-like structures on other fish rhabdoviruses-infected cells. PFR and EVEX infected-cells showed other rod-like or bubble-like structures on the surface, composed of membrane only.

Introduction

Viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV), pike fry rhabdovirus (PFR), eel virus of America (EVA) and eel virus of Europe (EVEX) are well known fish rhabdoviruses. Hirame rhabdovirus (HRV) is a fish rhabdovirus discovered from hirame, *Paralichthys olivaceus*, in 1984 (Kimura *et al.*, 1986). Characteristics of HRV have been found to be significantly distinguishable from other fish rhabdoviruses. This virus has been named *Rhabdovirus olivaceus* by Kimura *et al.* (1986), from the scientific name of the host species. In the comparative study of structural proteins of fish rhabdoviruses, HRV, IHNV and VHSV belonged to the genus *Lyssavirus* and PFR, EVA and EVEX belonged to the genus *Vesiculovirus* (Kimura *et al.*, 1989). In electron microscopical observation with negative staining, these virus particles were shown to have a bullet-shaped form and a membrane envelope. The size and shape of these virus particles were similar among the species (Zwillenberg *et al.*, 1965; Darlington *et al.*, 1972; Sano,

1976; Sano *et al.*, 1977). In this study, we compared observations on the ultrastructure of fish rhabdoviruses HRV, IHNV, PFR, EVA and EVEX *in vitro* using RTG-2 cells.

Materials and methods

Cell culture

RTG-2 cells (Wolf and Quimby, 1969) were cultured on the tissue culture plastic plate (diameter 25 mm) using Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum and 100 IU/ml penicillin and 100 µg/ml streptomycin. Before viral inoculation, these cells were incubated at 15°C for 24 hr.

Virus employed

Five species of fish rhabdoviruses were employed. These were HRV (8401-H) which was isolated from cultured hirame in Hyogo Prefecture, Japan in 1984; IHNV, provided by Dr. B.J. Hill (Fish Disease Laboratory, Ministry of Agriculture, Fisheries and Food, U.K.); EVA and EVEX, kindly provided by Dr. T. Sano (Tokyo University of Fisheries), and PFR, provided by Dr. P. de Kinkelin (Institute National de la Recherche Agronomique, France). Each virus were cultured on RTG-2 cells and the culture fluids were filtered and stored at -80°C until used.

Virus assay and electron microscopy

Each virus was diluted and inoculated into RTG-2 cell monolayers as m.o.i = 2. After absorption at 15°C for 60 min, the cells were washed three times with Hanks' BSS and fresh media was added.

For electron microscopical observation, infected cells were pre-fixed at 12 hr post-infection with 4% paraformaldehyde and 5% glutaraldehyde in a 0.1 M cacodylate buffer, pH 7.2 at 4°C for 3 hr. After pre-fixation, cell samples were post-fixed with 1% OsO₄ in the same buffer (4°C, 2 hr) and dehydrated through a graded series of ethanol and embedded in Epok 812 (Oken). Ultra-thin sections were cut on ultramicrotome with a sapphire knife (Sakura), and then stained with uranyl acetate and lead citrate. Preparations were observed with the H-7000 electron microscope (Hitachi) at 75 kv.

Results

HRV

At 12 hr post-infection, budding of virus particles appeared on the cell membrane, and sometimes many virus particles were observed in the intercellular spaces. Projections of a club-like structure were also found on the cell membrane with many virus particles budding from them (Fig. 1a). These structures revealed areas of high electron density and also

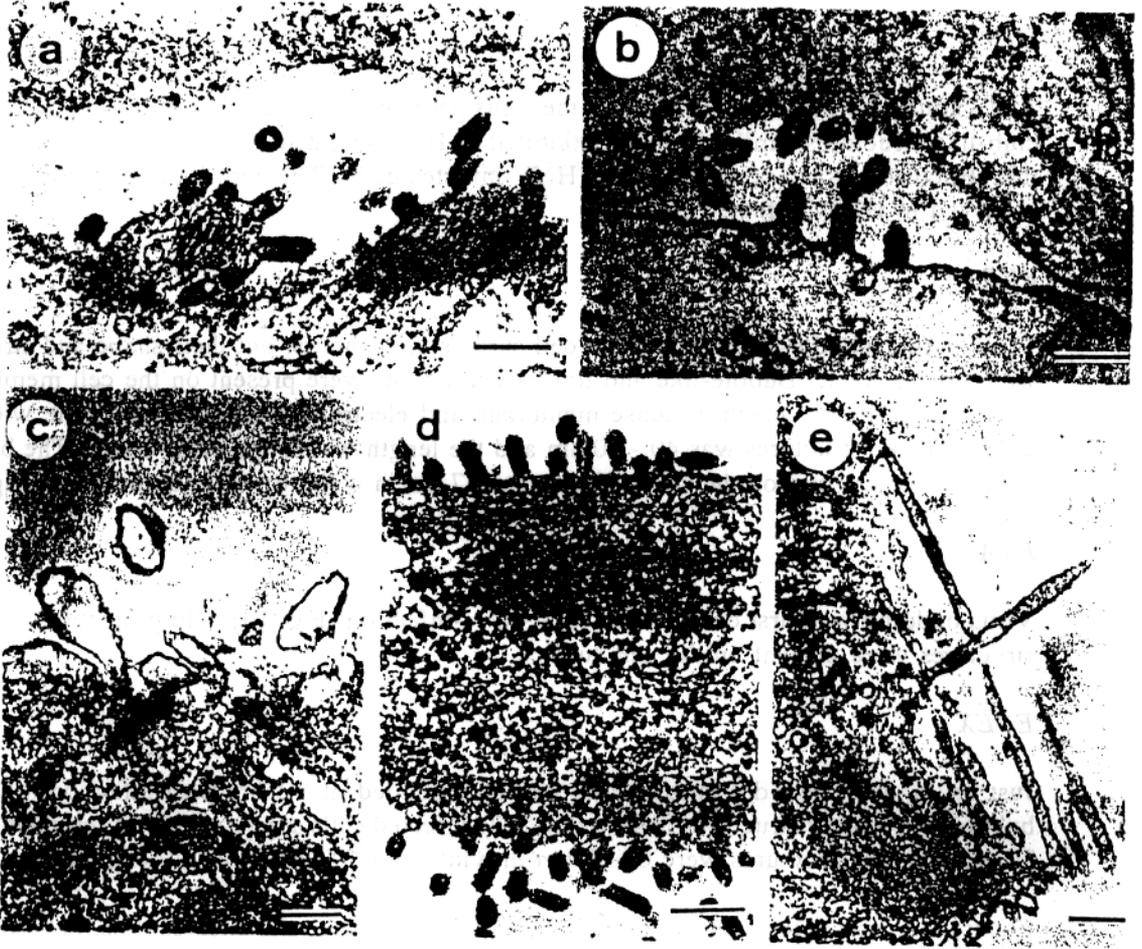


Fig. 1. Ultrastructural observation on fish-rhabdoviruses in RTG-2 cells at 12 hr post-infection. a). HRV-infected cells; club-like protuberances on the surface of cell, microfilament-like structure and viral particles budding from this protuberance (bar = 500 nm); b). IHNV-infected cells; viral particles budding from cell membrane and many other particles outside the cell (bar = 500 nm); c). PFR-infected cells; bubble-like structure on the surface of cell (bar = 500 nm); d). EVA-infected cells; viral particles budding from the cell membrane and at the outside of cell (bar = 500 nm); e). EVEX-infected cells; rod-like structure on the surface of cell (bar = 500 nm).

carried abundant microfilaments. These structures varied in size from 0.4 - 0.6 μm in width and were 0.8 μm long. Occasionally these structures even exceeded 1 μm in length and width. Other structures resembling very narrow and long cords of about 80 nm wide and about 3 μm long were also observed. Most of the variable projections, having club-like or cord-like structures, showed many virus particles on the surfaces.

IHNV

At 12 hour post-infection, budding of the virus particles on the cell membrane (Fig. 1b); and club-like structures similar to those exhibited in HRV-infected cells were found. However, this structure was not common in the IHNV-infected cells. The cord-like structures were not observed in IHNV-infected cells.

PFR

Virus particles were observed on the cell surface at 12 hours, but their shape and structure were not so clear. Bubble-like and rod-like structures were present on the cell membrane. They had a highly electron dense membrane and electron lucent contents. The width of these rod-like structures was 40 - 50 nm and the length was long, about 1 μm . The biggest bubble-like structure on the cell surface was 267 nm in width by 600 nm in length (Fig. 1c).

EVA

Virus budding was first observed at 12 hrs post-infection (Fig. 1d). No projections or other structures were present on the cell surface.

EVEX

Just as in other rhabdoviruses, virus particles budded at 12 hrs post-infection. Similar bubbles or rod-like structures observed in PFR-infected cells appeared on the cell membrane. These rod-like structures were 65-85 nm in width and 1.5 μm in length (Fig. 1e).

Electron microscopical observations of RTG-2 cells infected with different fish rhabdoviruses are summarized in Table 1.

Table 1. Electron microscopical observation of RTG-2 cells infected with different fish rhabdoviruses, HRV, IHNV, PFR, EVA and EVEX at 12 hrs post-infection.

Species	Size	Characteristics of the structure
HRV	80 x 180 nm	Club-like, cord-like protuberance
IHNV	80 x 160~180 nm	(Club-like protuberance)
PFRV	60~80 x 130~150 nm	Rod-like, bubble-like structure
EVA	55 x 160~180 nm	-
EVEX	60 x 160~180 nm	Rod-like, bubble-like structure

Discussion

Virus particle synthesis is known to be influenced by temperature and m.o.i. For VHSV, virus particles were reported to appear 24 hrs post-infection by Baroni *et al.* (1982), whereas De Kinkelin and Scherrer (1970) observed initial virus budding at 11 hrs post-infection. At m.o.i. = 2 and a temperature of 15°C, budding of viral particles of the fish rhabdoviruses employed appeared at about 12 hrs post-infection. These virus particles budded on the cell surface and were not observed in the vacuoles. Except for PFR, these viruses ranged in size from about 60-80 x 160-180 nm while PFR ranged from 60-80 x 130-150 nm. Our results were similar to the results of the negative staining described by Zwillenberg *et al.* (1965), Darlington *et al.* (1972), Fijan (1972), De Kinkelin *et al.* (1973), Cohen and Lenoir (1974), Olberding and Forest (1975), Sano (1976) and Sano *et al.* (1977).

PFR and EVEX-infected cells showed rod-like or bubble-like structures on the surface of the cell membrane which were significant only on the membrane. These structures resemble the empty virus envelopes external to the VHSV-infected cell membrane (Baroni *et al.*, 1982).

On the other hand, club-like or cord-like structures were easily observed in particular on the cell surface of HRV-infected cells. These structures were formed in many variations, but all showed high electron density and included microfilaments. Most of these structures showed a number of budding viruses on the surface. However, it is difficult to find these structures on the other examined fish rhabdoviruses-infected cells. Obviously, these structures are the most significant characteristics of the HRV-infected cells. It is well known that rabies virus, which belongs to genus *Lyssavirus*, has Negri bodies or Lyssa bodies in the cytoplasm (Matumoto, 1962; Matumoto *et al.*, 1974), and these were used for diagnoses of rabies virus by immunofluorescent or enzyme-immunoassay techniques (Coons and Kaplan, 1950). It is possible that if those structures on the HRV-infected cells are similar to Negri body, they may well be used for rapid diagnosis of HRV infection.

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