Herpesvirus cyprini: Biological and Oncogenic Properties

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In the present paper, we described the general biological properties and the oncogenicity of *Herpesvirus cyprini* or cyprinid herpesvirus 1 (CHV) (SANO, *et al.*, 1984) which were isolated from nine papilloma tissues developed on the skin or the caudal fin of Japanese carp.

The virus size were as follows; envelope diameter 190.4 ± 27.2 nm, projection length 20.5 ± 4.4 nm, capsid diameter 113.4 ± 8.6 nm. This herpesvirus showed good significant growth on FHM and MCT. The virus titer on FHM cells reached more than $10^{5.5}$ TCID₅₀/ml at 20°C incubation temperature for 14 days. Syncytium formation was not found in these cell lines. Both FHM and EPC cells infected with this virus possessed intranuclear inclusion, Cowdry type A. It occupied a relatively large area revealing rather irregular shape. The virus titer reduction of over 10^3 TCID₅₀/ml occurred before and after treatment with ether, pH 3, 50°C heating and IUDR.

A cluster of the virus particles was found inside and outside inclusion body in FHM cells infected with the virus. Virus particles were more numerous in the karyoplasm undergoing karyorrhexis. Budding immature virus particle from the nuclear membrane, as well as released virions in intracellular space, were observed.

Oncogenicity trial which we performed using common carp revealed grossly tumors on three fish out of ten. These tumor formations occurred more than five months after inoculation at 15.2°C of rearing temperature. The tumor cells induced in Asagi carp inoculated with the virus showed many virus particles in the karyoplasm and the cytoplasm.

The experimentally induced tumors on common carp and spontaneously-developed tumors on colored carp possessed the property to be desquamated at the natural condition.

Introduction

In Europe the papillomatous condition known as carp pox has been recognized since the Middle Ages (WOLF, 1983). The etiology was uncertain up to this time, but it is worthy of special mention that SCHUBERT (1964) provided the first viral evidence by means of electron micrographs. Although SONSTEGARD and SONSTEGARD (1978) tried to isolate any virus from the papilloma developed on American carp on FHM and other fish cell lines, they were not successful. However, they suggested that the papilloma could have been caused by virus since similar tumor appeared on the experimental fish which was kept together with the tumor bearing fish. Thus far, no one could isolate any viral agents directly from the tumor tissue, or from any other part of the host fish.

As reported in our previous paper (SANO *et al.*, 1984), we succeeded in isolating a herpesvirus from the papilloma tissue (Figs. 1 and 2) developed on

the skin and the caudal fin of fancy carp of the race called Asagi which had been raised in a commercial ornamental carp pond at Niigata Prefecture, in October of 1981. We designated this virus *Herpesvirus cyprini*. This paper describes more comprehensively the biological and oncogenic properties

Fig. 1. Gross appearance of the papilloma developed spontaneously on carp.

Fig. 2. Histological findings of the papilloma developed spontaneously. The tumor tissue is organized with well differentiated cells and epidermal peg interdigitations with papilla of dermal connective tissue which contains also vessel and others.

of the *Herpesvirus cyprini*.

Materials and Methods

Virus susceptible fish cell lines were investigated including 13 cell lines: EPC, MCT, FHM, CE-1 (Asagi Carp Embryo), RTG-2, BF-2, BB, SF (Sturgeon Fin), CHH-1 (Chum salmon Heart), TNK (*Tilapia nilotica* Kidney), SSE-5, EO (Eel Ovary), EK (Eel Kidney).

The virus was titrated on these cells incubated for 14 days at 20°C. Since the virus titer of *Herpesvirus cyprini* in FHM cells was higher than in EPC cells, we routinely used FHM cells for the subculture, and CE-1, MCT cells as well as FHM cells for a study on the viral antigen detection in cell inoculated with the herpesvirus. These cell lines were inoculated and incubated at 20°C. For the observation of the inclusion body, inoculated cells were stained with May Grünwald-Giemsa.

For infectivity trial, 1.5-year-old common carp, which were raised in Yoshida Training Center, Tokyo University of Fisheries, weighing 220 to 480 g, and average of 314 g, were inoculated intraperitoneally. The intraperitoneal dose per fish was 0.3–0.5 ml of virus $10^{5.4}$ TCID₅₀/ml, which was harvested in FHM cells. The same volume of Hanks' BSS was inoculated into each fish of different fish group. These groups were CHV harvested in FHM cells and control. Each group consisted of 10 fish. After injection, all fish of these groups

were raised separately in each trough with fluctuating temperatures of 13.0 to 16.5°C, an average of 15.2°C throughout the experimental period from July of 1983 to July of 1984.

A papilloma bearing Asagi carp was kept in an aquarium in our wet laboratory for long term observation of the tumor condition, from November of 1983 to June of 1984.

Prior to negative staining of the virus, the virus was cloned, grown in FHM cells and then the cultured fluid was concentrated by adding 6.5% PEG-6000 and NaCl to the final concentration of 0.4 M and stirring for 120 min at 4°C. The mixture was centrifuged at $2,000 \times g$ for 30 min at 4°C. The sediment was resuspended in Hepes buffered MEM-2 (pH 7.4) and centrifuged at $18,500 \times g$ for 30 min. The sediment was then resuspended in 0.005 M Tris (pH 7.4) containing 0.1% bovine serum albumin to 1/200 volume of the original virus solution. The virus was concentrated from $10^{4.8}$ to $10^{7.1}$ TCID₅₀/ml on the final procedure. The virus concentrated was negatively stained with 1% uranyl acetate or 2% PTA (pH 7.0) on copper grids. For ultrathin section of papilloma, the tumor tissue developed on the skin and the caudal fin of Asagi carp was prefixed for 50 min with cold 2.5% glutaraldehyde in 0.1 M phosphate buffer. The prefixed specimens were rinsed ten times for 20 min, each time with cold 7.5% sucrose in 0.1 M phosphate buffer solution and finally were kept for 12 h at 4°C. Subsequently the specimens were postfixed for one hour with cold 1% osmium tetroxide in PBS (–) after the sucrose solution was decanted. These specimens were dehydrated in alcohol, embedded in epoxy resin, sectioned and stained with 1% uranyl acetate for 30 min and with lead acetate (Millonig B method) for 15 min on copper grids.

IUDR inhibition test was carried out as follows: MEM-2 Hepes containing IUDR $10^{-3.7}$ M, MEM-2 Hepes containing IUDR $10^{-3.7}$ M plus 10^{-3} thymidine, and MEM-2 Hepes were provided. The virus solutions were diluted in serial 10-fold steps from 10^{-1} to 10^{-5} with each solution, and 0.05 ml of each mixture was inoculated into each well of eight wells provided for one step dilution which were seeded with 0.1 ml of FHM cells. Mock controls were prepared in the same way. These wells were incubated for 14 days at 20°C. NeVTA as the positive control and IPNV as the negative control, were prepared in the same procedure mentioned above.

They were inoculated into CHSE-214 cells and incubated 14 days at 15°C.

Acid (pH 3.0) susceptibility test was carried out as follows: 0.2 ml of the virus was added into 1.8 ml of MEM (pH 3.0) without NaHCO_3 and MEM adjusted to pH 7.2 by adding 0.01 M Tris buffer solution and reacted for three hours at 4°C agitating strongly. And then, after adjusted MEM (pH 3.0) without NaHCO_3 to pH 7.2 by adding 0.1 M Tris buffer solution, both the mixture were diluted 10-fold, from 10^0 to 10^{-4} , inoculated into FHM cells which was seeded in eight wells for each dilution, incubated for 14 days at 20°C and titrated.

Ether susceptibility test was performed as follows: 0.12 ml of ethyl ether was added into 0.5 ml of the virus and shooked vigorously for 18 h at 4°C. After evaporated the ether in the mixture by bubbling with N_2 -gas, the reacted virus was inoculated into FHM cells, incubated for 14 days at 20°C and titrated in the same way as in acid susceptibility test.

Heat stability test was carried out as follows: 0.5 ml of the virus heated at 50°C for 30 min was inoculated into FHM cells, incubated for 14 days at 20°C and titrated. The control virus was exposed at room temperature.

Results

CPE, in cell lines inoculated with the virus diluted more than 10^{-1} dilution, were limited to only FHM, EPC, MCT and CE-1 (Table 1). The virus titer in each cell line was as follows: $10^{5.8}$ TCID₅₀/ml in FHM, $10^{5.8}$ in MCT, $10^{3.8}$ in CE-1 and $10^{2.8}$ in EPC. There were characteristic differences in appearance of CPE among these four cell lines. In FHM cells, the infected cells revealed primarily vacuolation, in addition to roundness and also slight pyknosis in the focal area on the cell sheet 5 days after inoculation. Subsequently, CPE expanded from focal area to adjacent cells and the cellular desquamation at focal area occurred slowly. Cellular recovery at such area also was occasionally observed. About 30–40% cells still remained 2-weeks after inoculation. We recognized this condition as CPE completion in FHM cells. In MCT cells, CPE began to appear obscurely, but evenly over all cell sheet 5 days after inoculation and focal CPE was not remarkable. This was the contrasting difference between FHM and MCT cells. In MCT cells, the affected cells tended to lead to cellular

Table 1. CHV susceptible fish cell lines and effect of incubation temperature on cytopathic effect

Cell line	Temperature (°C)			
	10	15	20	25
FHM	—	++	+++	—
EPC	—	+	+	—
MCT	—	+	+++	—
CE-1	—	+	++	—
RTG-2	—	—	—	—
CHH-1	—	—	—	—
SSE-5	—	—	—	—
BF-2	—	—	—	—
BB	—	—	—	—
EO	—	—	—	—
EK	—	—	—	—
TNK	—	—	—	—
SF	—	—	—	—

—, Negative; +, weakly positive ($10^{2.8}$ TCID₅₀/ml); ++, positive ($10^{3.8}$ TCID₅₀/ml); +++, strongly positive ($10^{5.8}$ TCID₅₀/ml).

Fig. 3. Intranuclear inclusion bodies formed in FHM cells infected with *Herpesvirus cyprini*, which is interpreted as Cowdry type A. Stained with May Grünwald-Giemsa.

collapse after they became swollen or became round without cellular desquamation. In both EPC and CE-2 (Kohaku Carp Embryo) cells, CPE is less remarkable than FHM and MCT. In comparison with the control cell, the infected EPC and CE-2 cells did exhibit some cellular roundness and desquamation. CPE in both cell lines began to appear around 10 days after inoculation. However, immunofluorescent antibody technique showed that 5th day after inoculation the viral antigen existed only in the karyoplasm of CE-2 cells inoculat-

ed with *H. cyprini* and incubated at 20°C. Syncytium formation in the present study also was not found in FHM and EPC cells. However, FHM cells which were infected with this herpesvirus showed advanced CPE, and possessed intranuclear inclusion which were interpreted as Cowdry type A. In many cases, it was surrounded by a halo (the space between the inclusion and the nuclear membrane often appeared as non-stained area with May-Grünwald Giemsa) and occupied a relatively large area in the karyoplasm, revealing somewhat irregular shape (Fig. 3).

The virus titer was 10^{5.8} TCID₅₀/ml in FHM and has been remained at that level in FHM cells thus far.

As shown in Table 2, the virus titer reduction of significantly large difference over 10³ TCID₅₀/ml occurred regardless of any treatment used.

This herpesvirus has possessed comparatively long filamentous projections, and were thicker at top. It is different from spike-like NeVTA (SANO, 1976) found on the surface of the envelope. The envelope of *H. cyprini* showed membrane-like structure (Fig. 4). The tegument of this virus was considerably less than the herpesvirus YTV (SANO *et al.*, 1983) isolated from the basal cell epithelioma tissue on Yamame, *Oncorhynchus masou* and

possessed low electron-density. Dimension in each portion of this virus as shown in Table 3.

The relative comparison of the envelope between *H. cyprini* and NeVTA showed that the size of former's envelope was significantly smaller than the NeVTA's envelope. *H. cyprini* profile was irregular round shape and seemed to be similar to HSV.

Many virus particles were observed in thin sectioned tumor cells of the papilloma developed on Asagi carp inoculated with *H. cyprini*. Particularly, the virus particles were more in the cytoplasm showing diverse virus profiles (Fig. 5). In the nucleus, also the diverse viral particles and several virogenic stages were observed. The tumor cells in which the virus particles were found, generally revealed the reduction in staining of mitochondria, endoplasmic reticulum and other organella or dis-

Table 2. Titer reduction of CHV after treatment with ether, pH 3, heat and IUdR

Treatment	Virus titer in log TCID ₅₀ /ml	
	Before treatment	Reduction
Ether	3.9	> 3.1
pH 3	4.5	> 3.8
Heat	3.8	> 3.0
IUdR	5.1	> 4.3

Table 3. Dimension of CHV virion

Portion	Range (nm)	Average (nm)
Envelope in diameter	153.5–239.9	190.4 ± 27.2 (n = 42)
Projection in length	20.5–37.2	29.8 ± 4.4 (n = 12)
Capsid in diameter	93.0–125.6	113.4 ± 8.6 (n = 94)

Fig. 4. The profile of *H. cyprini*. Note comparatively long projections which are filamentous with thicker at top on the surface of the envelope (bar = 100 nm).

Fig. 5. Many herpesvirus particles found in the cytoplasm of thin sectioned tumor cell of the papilloma developed on the skin of the Asagi carp inoculated with the *H. cyprini* (bar = 300 nm).

Fig. 6. Appearance of many viral particles, reduction in staining of mitochondria, endoplasmic reticulum and etc., disappearance of such organelles. The intranuclear inclusion can be found in such tumor cell. Some virus particles occluded (arrow) in the intranuclear inclusion and a cluster of the virus particles released outside the inclusion. IC indicates inclusion (bar = 1 μ).

Fig. 8. Virus particles which are touched on or penetrated with filament-like materials derived from the area with high electron-density. It seems that the nucleocapsid is going to be equipped with such materials (bar = 300 nm).

Fig. 7. Virus particles occluded in an intranuclear inclusion in FHM cell infected with *H. cyprini* (bar = 500 nm).

Fig. 9. Virus particles appeared numerous in the karyoplasm undergoing karyorrhexis in FHM cells infected with *H. cyprini* (bar = 300 nm).

appearance of such organella. Noticeable reduction of staining was observed in the inner area of the nucleus of the cell in which constructing viral particles were frequently found (Fig. 6). Intranuclear inclusion was also recognized in the tumor cells mentioned above. It showed rather irregular shape with low electron-density and occluded a few virus particles. A cluster of the virus particles was found simultaneously outside the inclusion (Fig. 6). Figure 7 also shows the virus occlusion in an intranuclear inclusion appeared in FHM cells infected with *H. cyprini*. In thin-sectioned FHM cells inoculated with this virus, the virus particles were generally found less in the

Fig. 10. Budding virus particles from the nuclear membrane and released virions into intracellular space. An arrow indicates budding. Two arrows indicate released. Three arrows indicate capsid (bar = 300 nm).

cytoplasm while they existed numerous in the karyoplasm. Diverse figures we observed in the karyoplasm may help to understand the virogenic process. Namely, many different viral particles assembled to a certain area of the karyoplasm showed low electron-density and the area surrounding those particles showed high density. We found out that

Fig. 11. A tumor developed on the right pectoral fin of the experimental common carp inoculated with *H. cyprini*. It was found six months after inoculation.

some particles were surrounded, touched by or penetrated with filament-like materials (Fig. 8). These filament-like materials having diverse length and radial arrangement in high density, were found throughout the karyoplasm (Fig. 8). Virus particles were more numerous in the karyoplasm undergoing karyorrhexis (Fig. 9). Budding virus particles from the nuclear membrane, were observed (Fig. 10).

In Table 4, the results of the oncogenicity trial are shown. No mortality was recorded since we set up the experiment and through April 30, 1984, either in the experimental group and the control group. However, the experimental group which was inoculated with CHV harvested in FHM, disclosed

Table 4. Incidence of the papilloma bearing experimental fish found six months after inoculation by intraperitoneal injection into common carp with CHV

Control	CHV harvested in FHM
0/10	3/10

Fig. 12. Two tumors developed spontaneously on the dorsal skin (a) and the caudal fin (b) of Asagi carp. Photographed in March, 1984.

Fig. 13. Entirely spontaneously desquamated two tumors on the same fish as shown in Fig. 12. Photographed June 15, 1984.

the tumor formation in three out of ten fish.

We found a tumor formation on the right pectoral fin of the experimental carp which had been inoculated with *H. cyprini* on January 31, 1984 (Fig. 11). It took more than five months after inoculation to develop the visible tumor at average water temperature of 15.2°C. These tumors desquamated spontaneously before tumor tissue had developed enough for sample to be collected for both histological and virological studies.

Our observation indicated that all papilloma which developed naturally on the caudal fin and the dorsal skin of one Asagi carp, desquamated spontaneously. We had traced the tumor condition of one Asagi which was kept in our aquarium at average water temperature of 16.4°C (November, 1983), 11°C (January to March, 1984) and 22.0°C (June, 1984) (Figs. 12 and 13). The desquamated lesions were smooth and not hemorrhagic.

Discussion

We succeeded in isolating *Herpesvirus cyprini* or cyprinid herpesvirus 1 from the papilloma tissue on the caudal fin and the skin of nine carp, which included one tumor tissue desquamated from the skin into water, over the two years period from October, 1981 to November, 1983 in Japan.

We found some similarities between the virus profile in ultrathin section of *H. cyprini* and that which SCHUBERT (1964, 1966) showed for carp pox virus in ultrathin sections. However, neither SCHUBERT nor SONSTEGARD and SONSTEGARD (1978) were able to isolate the virus of carp pox.

Of course, *H. cyprini* was entirely different from CCV (WOLF and DARLINGTON, 1971), NeVTA (SANO, 1976), *H. salmonis* (WOLF *et al.*, 1978), OMV (KIMURA *et al.*, 1981), YTV (SANO *et al.*, 1983) and *H. vitreum* (KELLY *et al.*, 1983) which have already been isolated.

In the present paper, we described the general biological properties of *H. cyprini* and its oncogenicity. The results obtained from the oncogenicity trial, which we have performed using common carp in this study, verified that this herpesvirus experimentally reproduced naturally occurring tumor, these supporting the results in our previous study (SANO *et al.*, 1984). Consequently, we came to the conclusion that *Herpesvirus cyprini* may be the causative agent of carp papilloma.

Although the term carp pox has been used for many years, it is a misnomer because the lesions are not really pox. Similarly, *epithelioma papulosum* is not a proper designation because neither the spontaneously occurring tumor nor the experimentally induced tumor (SANO *et al.*, 1985) possess the histological characteristics of the basal cell epithelioma of *O. masou* described by SANO *et al.* (1983). Since the lesions possess characteristics of papilloma, we propose that the term *papillosum cyprini* be used hereafter instead of carp pox or *epithelioma papulosum*.

Future studies are anticipated on whether this herpesvirus is pathogenic to the other cyprinids and to the juvenile fishes. Past studies on OMV and YTV had revealed the high pathogenicity of the viruses to juvenile of some salmonids. Studies on the molecular characterization of *Herpesvirus cyprini* is also planned.

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