

## Establishment and Characterization of a Cell Line Persistently Infected with Infectious Pancreatic Necrosis Virus (IPNV)

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**Meei-Mei Chen, Guang-Hsiung Kou and Shiu-Nan Chen (1993)** Establishment and characterization of a cell line persistently infected with Infectious Pancreatic Necrosis Virus (IPNV). *Bull. Inst. Zool., Academia Sinica* 32(4): 265-272. The present study characterizes a new cell line (designated SB) established from the swim bladder of the black carp, *Mylopharyngodon piceus*. The SB cell line lost contact inhibition with high plating efficiency and anchorage independence from the beginning of the primary culture. Icosahedral viral particles and inclusion bodies were found in the cytoplasm of some SB cells which lacked viral inoculation, suggesting that the viruses originated from persistent viral-infected cells of the fish. After treatment with chloroform and the metabolic inhibitor 5-fluoro-2'-deoxyuridine (FUDR), the virus proved to be a non-enveloped RNA virus. Results from an analysis of purified viral nucleic acid also indicated that this virus was an RNase A resistant, bi-segmented double-stranded RNA virus. The supposition that the virus was a mutant of the Infectious Pancreatic Necrosis Virus (IPNV) Ab serotype arose after the presumptive serotyping of the viral antigen by both polyclonal and monoclonal antibodies.

**Key words:** Black carp (*Mylopharyngodon piceus*), Infectious Pancreatic Necrosis Virus, Viral persistent infected cell line.

*Mylopharyngodon piceus*, the black carp, is a snail-feeding fresh water fish which, as a result of its rapid growth rate, flavor, and high nutritional content, has become an intensively cultured fish species in several parts of China. We attempted to establish a black carp cell line for the purpose of viral research. One cell line, from a black carp swim bladder (designated swim bladder (SB) cell), was identified as an infectious pancreatic necrosis virus (IPNV) persistently infected (PI) cell line.

IPNV is a pervasive pathogen in Taiwan. It has been isolated in many species of fish including: Japanese eels (*Anguilla japonica*), tilapia (*Tilapia mossambica*), rainbow trout (*Oncorhynchus mykiss*), milkfish (*Chanos chanos*), loach (*Misgurnus anguillicaudatus*), and perch (*Lateolabrax japonica*) (Chen et al. 1985, Hedrick et al. 1983, Lipipun et al. 1989). Survivors of an IPNV-infected fish culture usually become lifelong carriers, and spread the virus through feces and seminal fluid (Billi and Wolf 1969). Carrier fish are asymptomatic, and cause either horizontal

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or vertical infection of other fish. The propagation of IPNV by carrier fish is a severe problem in aquaculture; consequently, carrier fish detection and elimination are urgent tasks.

Three IPNV persistently-infected cell lines (chinook salmon embryo cell (CHSE-214), steelhead trout cell (STE-137), and rainbow trout gonad cell (RTG-2)) have been established in vitro (Hedrick and Fryer 1981 1982, MacDonald and Kennedy 1979). Persistently-infected and uninfected cell lines are indistinguishable by morphology and growth characteristics. Similar to reported IPNV PI cell lines, SB cells can release IPN infectious virus and resist homologous virus superinfection, but they are susceptible to a heterologous virus challenge; therefore, it has been suggested that the SB cell line is an IPNV PI cell line. The SB cell line showing characteristics of viral-persistent infection was not established by artificial viral infection; instead, it was derived from the natural carrier.

## MATERIALS AND METHODS

### Primary culture

A black carp donated by the Jwu Bei Branch Station, Taiwan Fisheries Research Institute, was used for the present study. The fish exhibited clinical symptoms of ulceration and hemorrhaging on its skin, fins, and liver. The heart, liver, spleen, ovary, and swim bladder were washed thoroughly and minced into approximately 1 mm<sup>3</sup> fragments, then transferred to flasks. The culture medium contained Leibovitz L-15 medium (L-15) supplemented with 20% fetal bovine serum (FBS, Gibco, Melbourne, Australia) and 10% cultured medium derived from a color carp testis cell line (CCT) (Ku and Chen 1992), and then filtered through 0.2 µm filter. Antiseptic compounds—including

200 I.U. penicillin, 200 µg streptomycin, and 50 µg fungizone per ml of solution—were also introduced. The primary culture was incubated at 28°C.

### Cell morphology

SB cells at subculture 10 were processed for transmission and scanning electron microscopic observation as described by Moss and Gravell (1969) for morphological characteristic investigation. Additionally, the SB cell line growing on cover slips was stained with acridine orange as per Humason (1979).

### Virus isolated from SB cell characterization

Cultured SB cell medium was incubated with either chloroform or FUDR, and activity was detected via procedures described by Rovozzo and Burke (1973) to investigate the effects of chloroform and 5-fluoro-2'-deoxyuridine (FUDR) on the activity of these viral particles. Virus genome type was determined by incubation-purified viral genome with RNase A at 37°C for 1hr, followed by electrophoresis (Hedrick et al. 1985).

### Western immunoblot

Isolated virus serotype was determined by a Western immunoblot assay following procedures described by Chi and Chen (1991).

## RESULTS

### Primary cell culture

Cells derived from the heart, liver, spleen, and ovaries failed to grow because of bacterial contamination (septicemia). However, swim bladder fragments were not

contaminated, and therefore tissue fragment adherence was observed. Approximately 15 days after inoculation, cells migrated quickly from these swim bladder fragments. Three days later, the cells migrating from these fragments began to accumulate, peel off, and form cellular masses in the culture medium before a monolayer was formed. These cells were subcultured at a split ratio of 1:3. After being transferred into new flasks, tissue fragments and suspended cellular masses immediately adhered to both the flask and cells, and cell masses continued to migrate from the tissue fragments. Cell mitosis was frequently observed. The propagation of these cells (designated as SB cells) was so rapid that fetal bovine serum (FBS) concentration diminution was considered. The FBS concentration in medium was consequently reduced from 20% to 3%.

### Morphological observations

Under a phase contrast microscope the SB cells showed a multi-type morphology, including fibroblast-like cells, epithelioid cells, giant cells with multiple nuclei, and cells with dentrite-like or ramified protrusions (Fig. 1).

Using acridine orange, multiple yellowish-green nuclei and yellowish-green viral inclusion bodies were observed in the cytoplasm (Fig. 2). The yellowish-green viral inclusion bodies implied that the viruses present in the cytoplasm were double-stranded nucleic acid viruses (Dimmock and Primrose 1987).

Observed under a transmission electron microscope, the SB cells revealed severe vacuolation in the cytoplasm. Viral inclusion bodies and free viruses were also observed (Fig. 3). Unidentified densely-stained particles were frequently found in the distended nuclear membrane. The mitochondria of many SB cells were dilated and densely

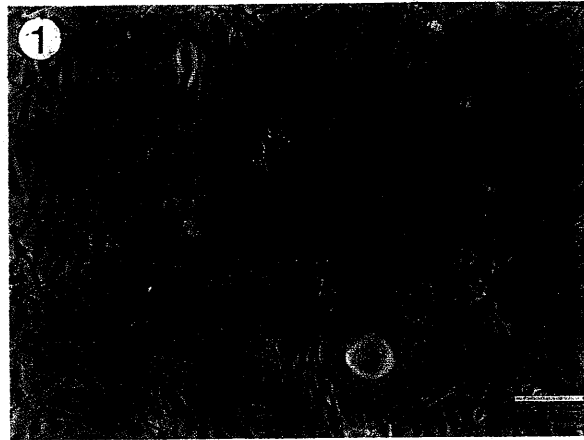


Fig. 1. Morphology of the SB cell line under the phase contrast microscope. x100



Fig. 2. Acridine orange-stained SB cells. One giant cell with multiple yellowish-green nuclei located in the center (arrowhead) and many cytoplasmic yellowish-green inclusion bodies can be seen (arrow). Original magnification x 400.

stained. Thin sections of the kidney and spleen from the carrier fish were observed under a transmission electron microscope. The kidney cells exhibited many morphological similarities to the cultured cells, including a distended nuclear membrane and mitochondria. Virus-like particles were observed in the cytoplasm (Fig. 4).

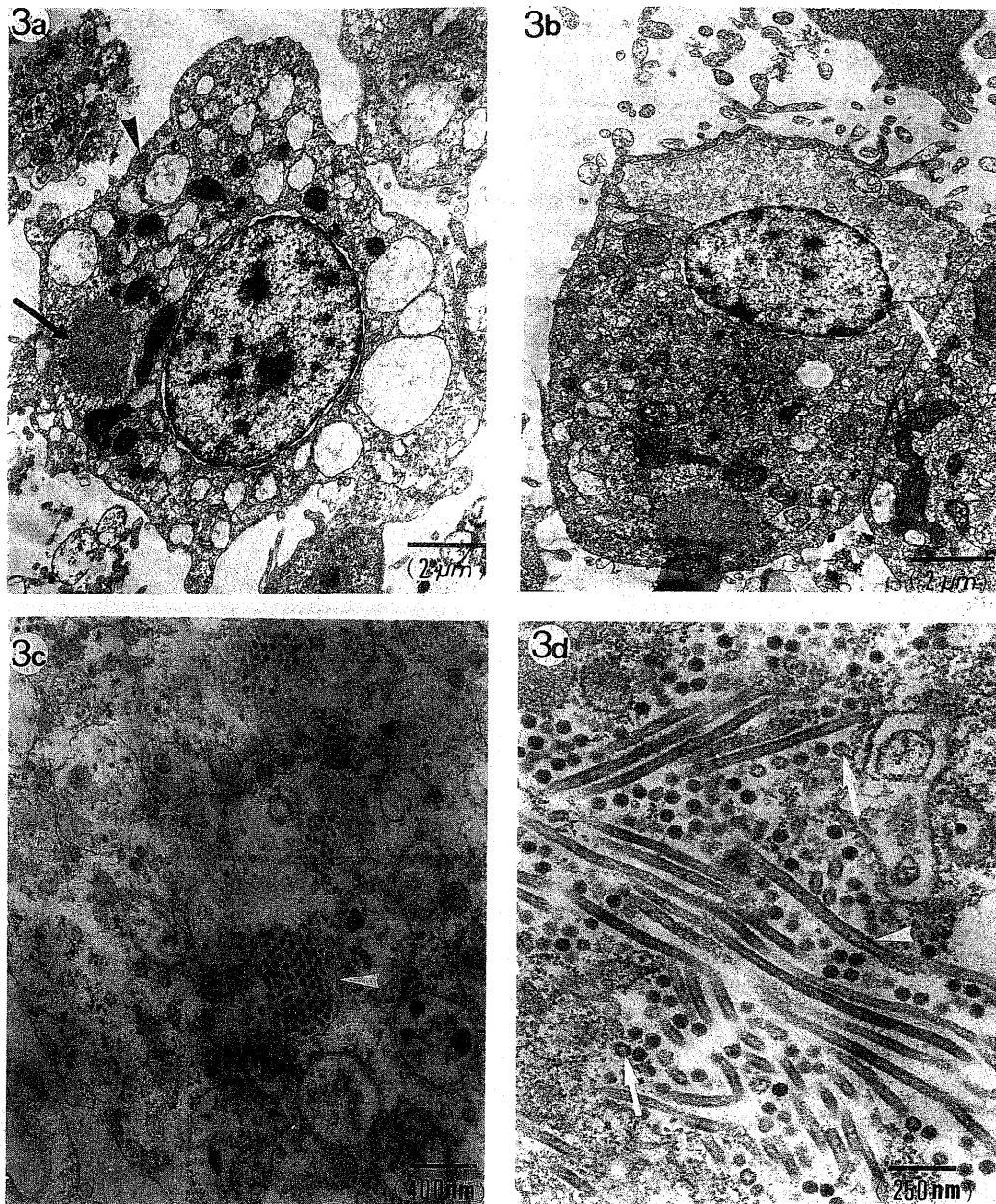


Fig. 3. Transmission electron micrographs of an SB cell. a: The SB cell exhibited severe vacuolation of cytoplasm. An SB virus-containing body (arrowhead) and viroplasm (arrow) were also observed. x6000. b: Dilated nuclear membrane (arrow) containing unidentified particles (arrowhead) were observed. x6000. c: Viral inclusion bodies (arrowhead), x25,000. d: Free viral particles, viral protein coats (arrow), and microtubules of this cell (arrowhead), x40,000, present in the cytoplasm of the SB cell.

### Characteristics of the SB Virus

The SB cell cultured medium had titer of  $10^{7.67}$  TCID<sub>50</sub>/ml,  $10^{7.57}$  TCID<sub>50</sub>/ml to TO-2

cells (Chen et al. 1983), and CCT cells, respectively. The optimal infection temperature for the virus in the TO-2 and CCT cells was 18°C. Neither chloroform nor FUDR had

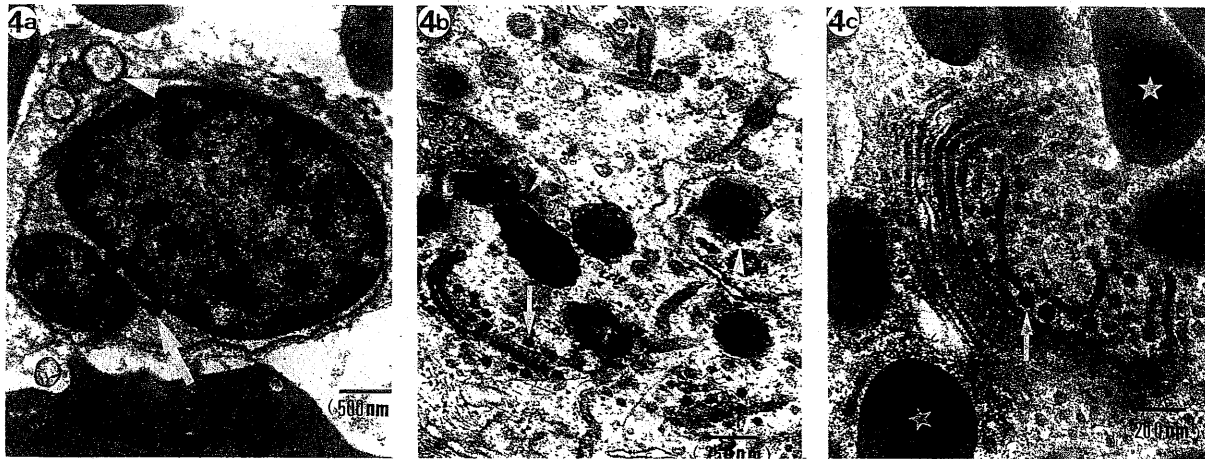


Fig. 4. Transmission electron micrographs of an SB cell-carrier fish kidney. a: Interstitial cell of kidney showed vacuolation in the cytoplasm (arrowhead) and dilated nuclear membrane containing unidentified particles (arrow). x20,000. b: Virus-like particle (arrow) near golgi bodies; the organelles of this cell were degraded and densely stained (arrowhead). x40,000 c: Secreting granules (star) and virus-like particles (arrow) presented near golgi body. x50,000.

Table 1. TCID<sub>50</sub> of SB cell-cultured medium incubated with chloroform and FUDR

Treatment	Chloroform	FUDR	PBS buffer
TCID <sub>50</sub>	10 <sup>7.43</sup>	10 <sup>7.75</sup>	10 <sup>7.5</sup>

TCID<sub>50</sub> detected in CCT cells.  
FUDR: 5-fluoro-2'-deoxyuridine.

significant effects on viral activity when compared with the control (PBS) (Table 1). An electrophoresis of the viral RNA showed that the viral genome contained two segments and was resistant to RNase A (data not shown). These results, in conjunction with the results given in Fig. 2, indicate that the SB cell-isolated virus was infectious pancreatic necrosis virus. We named this virus IPNV SB virus (SB virus). When electrophoresed in a 10% polyacrylamide gel, the SB viral RNA comigrated with the IPNV Ab serotype (Fig. 5, lane 5) but differentiated from Sp RNA (Fig. 5, lane 6).

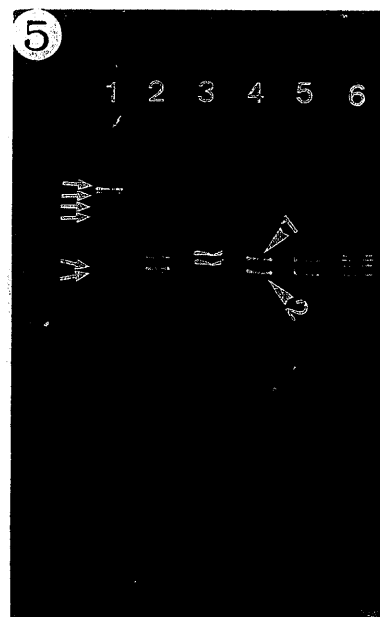


Fig. 5. Electrophoresis of SB viral RNA in 10% polyacrylamide gel. Lanes 1-6 are DNA/Hind III fragment markers (BRL) (arrows from top to bottom are 23.1Kb, 9.4Kb, 6.5Kb, 4.3Kb, 2.3Kb and 2.0Kb), IPNV Ab serotype RNA, IPNV Sp serotype RNA, SB viral RNA (arrowhead 1: A segment; arrowhead 2: B segment), IPNV Ab serotype RNA mixed with SB viral RNA, and IPNV Sp serotype RNA mixed with SB viral RNA.

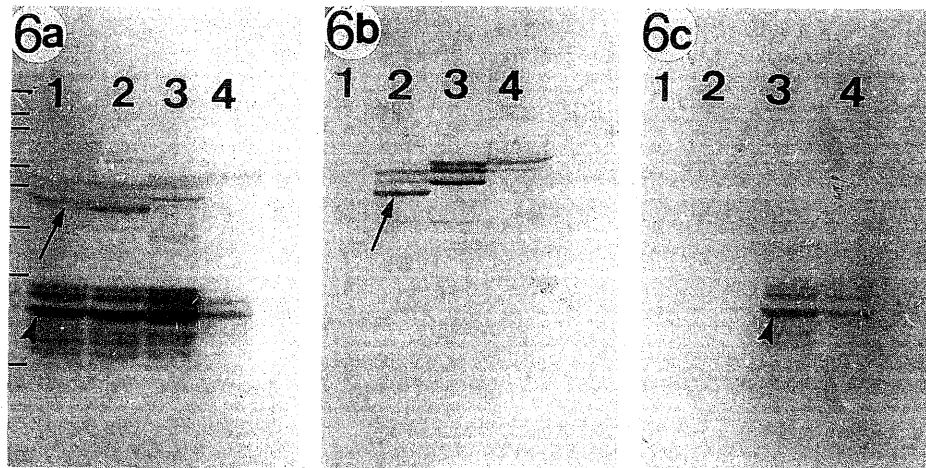


Fig. 6. Western immunoblot of viral antigens which reacted with IPNV polyclonal and monoclonal antibodies. a: Viral antigens reacted with a rabbit anti-IPNV Ab serotype polyclonal antibody. b: Viral antigens reacted with an Eb1 (anti-VP2 specific) monoclonal antibody. Lanes 1-4 of a. and b. are: SB virus, Ab, Sp, and VR299. c: Viral antigens reacted with an Er1 (Ab serotype-specific anti-VP3 specific) monoclonal antibody. Lanes 1-4 are VR299, Sp, Ab and SB virus. The molecular weights of the black lines from top to bottom were 200, 116.3, 97.4, 66.3, 55.4, 36.5, 31.0, 21.5 Kd, respectively (wide-range protein standards of novel experimental technology). arrow: VP2 polypeptide. arrowhead: VP3 polypeptide.

### Western immunoblot

A Western immunoblot of a viral antigen which was derived from virally-infected lysed cellular debris against rabbit anti-IPNV Ab serotype polyclonal antibody revealed that this virus has a different VP2 antigenic pattern to the infectious pancreatic necrosis virus (IPNV) Ab, Sp, and VR299 serotypes (Fig. 6a). The  $R_f$  value of the VP2 antigenic group of this virus was between the Ab and Sp serotypes. Monoclonal antibodies against eel virus of European (EVE) were used to identify the relationship of the SB virus to these two IPNV serotypes (Chi and Chen 1991). SB viral antigens showed no precipitation line with the VP2 specific monoclonal antibody, Eb1 (which was previously demonstrated to be able to react with Ab, Sp, and VR299 serotypes) (Chi and Chen 1991) (Fig. 6b). In contrast, precipitation lines were observed between the SB viral antigens and the Ab serotype-specific anti-VP3 monoclonal

antibody (Er 1 monoclonal antibody) (Fig. 6c). Precursor and decay polypeptide VP2 and VP3 bands were observed in these cell-associated antigens.

### DISCUSSION

The SB cell line derived from the studied black carp swim bladder was established for the present study. Differing from other IPNV persistently-infected cell lines by its lack of in vitro inoculation, this SB cell line was derived from persistent-virally infected fish. Although we did not find any concrete evidence to prove this, experimental information supports this assertion. First, the CCT cultured medium (which was a component of the primary culture medium) was virus-free. Second, in the IPNV persistently-infected (PI) cell lines, the persistence of IPNV was induced in vitro, and the propagation of PI-cells began from the survivors of

the IPNV infected cells (Hedrick and Fryer 1981 1982, MacDonald and Kennedy 1979). The SB cell was not derived from the IPNV-infected cell survivors, but it migrated and propagated directly from the tissue fragments. Third, the SB cell line exhibited loss of contact inhibition, anchorage-independence, high plating efficiency, and low serum requirements at the beginning of the primary culture, which demonstrates that the transformed SB cell characters originated directly from the tissue fragments. Moreover, we observed many morphological similarities between the SB and kidney interstitial cells, including a dilated nuclear membrane, and enlarged densely-stained mitochondria, endoplasmic reticulum, and cytoplasmic vacuoles. These characteristics can be taken as showing the effect of IPNV SB virus persistence. Consequently, the persistence of the SB virus originated from carrier fish.

The SB virus is characterized by bisegmented double-stranded RNA and a medium size (about 60 nm), unenveloped, and single-layer icosahedral capsid. This virus precipitated with both the IPNV Ab serotype polyclonal and Ab serotype specific anti-VP3 polypeptide monoclonal antibodies, but not with the anti-VP2 polypeptide specific monoclonal antibody (anti-VP2 polypeptide of Ab, Sp, and VR299 serotypes of IPNV). The molecular weight of the VP2 polypeptide was about 50 KD—higher than the VP2 polypeptide of the Ab serotype, but lower than the VP2 polypeptide of the Sp serotype. These results suggest that the observed SB virus is an IPNV Ab serotype mutant. A mutation at the VP2 polypeptide resulted in an antigenic SB virus variation which might have lead to a persistent infection of the host.

Two IPNV persistent infection mechanisms have been previously reported (Hedrick and Fryer 1981, Okamoto et al. 1983). One was interferon, and the other was defective

interfering (DI) particles. In the SB cell, interferon could not be detected, so the SB cell was susceptible to challenges from reoviruses and herpes viruses, which produced a completely cytopathic effect. But the exact role of interferon in the persistence of the SB virus needs further research. Regarding the defective interfering (DI) particle, the IPNV DI particle showed an RNA deletion in the large segment (A segment) and produced a smaller VP2 polypeptide than did the standard virus (Lo et al. 1990 1991). The SB virus had a larger VP2 polypeptide than the standard Ab serotype. Therefore, the SB virus persistent infection was not an effect of a DI particle. We inferred that the mutation of the VP2 polypeptide may influence the SB cell cytopathic effect and induce SB virus persistence, but the exact mutation mechanism requires further study.

## REFERENCES

- Billi JL, K Wolf. 1969. Quantitative comparison of peritoneal washes and feces for detecting infectious pancreatic necrosis (IPN) virus in carrier brook trout. *J. Fish Res. Can.* **26**: 1459-1465.
- Chen SN, SC Chi, Y Ueno, GH Kou. 1983. Establishment of a cell line from ovary of tilapia. *Bull. Europ. Asso. Fish Pathol.* **3**: 1-4.
- Chen SN, GH Kou, RP Hedric, JL Fryer. 1985. The occurrence of viral infections of fish in Taiwan. *Fish and Shellfish Pathol.* pp. 313-319.
- Chi SC, SN Chen. 1991. Establishment, characterization and application of monoclonal antibodies against eel virus European (EVE). *Fish Pathol.* **26(1)**: 1-7.
- Dimmock NJ, SB Primrose. 1987. Introduction to Modern Virology. Volume 2 of Basic Microbiology, ed. J.F. Wilkinson. Third edition. Blackwell Scientific Publications. London: Oxford, pp. 333.
- Hedrick RP, JL Fryer. 1981. Persistent infection of three salmonid cell lines with infectious pancreatic necrosis virus (IPNV). *Fish Pathol.* **15**: 163-172.
- Hedrick RP, JL Fryer. 1982. Persistent infections of salmonid cell lines with infectious pancreatic necrosis virus (IPNV): A model for the carrier state in trout. *Fish Pathol.* **16**: 164-172.

- Hedrick RP, JL Fryer, SN Chen, GH Kou. 1983. Characteristics of four birnaviruses isolated from fish in Taiwan. *Fish Pathol.* **18**: 91-97.
- Hedrick RP, WD Eaton, JL Fryer, YC Yeh, JW Park, SW Hong. 1985. Biochemical and serological properties of four birnaviruses isolated from fish in Korea. *Fish Pathol.* **20**: 463-468.
- Humason GL. 1979. *Animal tissue techniques*, 4th ed. San Francisco: WH Freeman and Company, pp. 465-467.
- Ku CC, SN Chen. 1992. Characteristics of three cell lines derived from color carp (*Cyprinus carpio* L.). *J. of Tissue Culture Methods* **14(2)**: 63-72.
- Lipipun V, P Caswell-Reno, YL Hsu, JL Wu, MC Tung, PW Reno, W Wattanavijarn and BL Nicholson. 1989. Antigenic analysis of Asian aquatic birnavirus isolates using monoclonal antibodies. *Fish Pathol.* **24(3)**: 155-160.
- Lo CF, MS Lin, SM Liu, CH Wang, GH Kou. 1990. Viral interference in TO-2 cells infected with IPN virus isolated from clam, *Meretrix lusoria*. *Fish Pathol.* **25**: 133-140.
- Lo CF, CH Wang, MS Lin, GH Kou. 1991. Viral polypeptides and RNA of defective interfering particles of IPN virus generated by serial undiluted passaging in TO-2 cells. *Fish Pathol.* **26**: 119-125.
- MacDonald RD, JC Kennedy. 1979. Infectious pancreatic necrosis virus persistently infects Chinook salmon embryo cells independent of interferon. *Virology* **95**: 260-264.
- Moss HL, M Gravell. 1969. Ultrastructure and sequential development of infectious pancreatic necrosis virus. *J. Virol.* **3**: 52-58.
- Nicholson BL. 1975. Possible interference in the isolation of IPN virus from carrier fish. *J. Fish Res. Bd. Can.* **32**: 1437-1439.
- Okamoto N, T Shirakura, Y Nagakura and T Sano. 1983. The mechanism of interference with fish viral infection in the RTG-2 cell line. *Fish Pathol.* **18**: 7-12.
- Reno PW, S Darley and M Savan. 1978. Infectious pancreatic necrosis: Experimental induction of a carrier state in trout. *J. Fish Res. Bd. Can.* **35**: 1451-1456.
- Rovozzo GC, CN Burke. 1973. *A manual of basic virological techniques*. Englewood Cliffs, NJ: Prentice-Hall, pp. 146-151.

## 魚類傳染性胰臟壞死病毒持續性感染細胞株之建立及特性研究

陳嫩玫 郭光雄 陳秀男

本篇論文報告一病毒持續性感染細胞株之建立及其特性，此細胞株源自青魚 (*Mylopharyngodon piceus*) 鰾組織被命名為SB細胞。SB細胞在初級細胞培養時期即表現出喪失停泊依賴性及接觸依賴性，血清依賴性降低，群落形成率高等轉型細胞特性。在電子顯微鏡下可觀察到有些細胞的細胞質內存在有二十面體病毒顆粒及病毒包涵體，而此病毒源自此魚魚體。SB細胞釋放出來的病毒經由氯仿及代謝抑制物的處理及分析得知此病毒為不具外套膜的RNA病毒。純化的病毒核糖核酸以RNase A處理並經電泳分析得知此病毒為抗RNase A的兩段式雙股核糖核酸病毒科的傳染性胰臟壞死病毒 (Birnaviride, Infectious Pancreatic Necrosis Virus, IPNV)，再經免疫轉印法以多源及單源抗體比較此病毒與IPNV之Ab, Sp, VR299三種血清型病毒抗原之異同，得知此病毒不同於這三種血清型且應為IPNV Ab血清型之變異株。