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Establishment and characterization of a continuous cell line (GF-1) derived from grouper, *Epinephelus coioides* (Hamilton): a cell line susceptible to grouper nervous necrosis virus (GNNV)

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Abstract

A new continuous cell line (GF-1) was established and characterized. The GF-1 cell line, derived from the fin tissue of a grouper, *Epinephelus coioides* (Hamilton), was maintained in L15 medium containing 5% foetal bovine serum (FBS) at 28 °C, and has been subcultured more than 160 times since 1995. The majority of GF-1 cells are fibroblast-like, together with some epithelioid cells. Spontaneous transformation of GF-1 cells occurred during subculture 50 to subculture 80, and led to an increase of plating efficiency, less requirement of FBS and *de novo* susceptibility to grouper nervous necrosis virus (GNNV). Cytopathic effects (CPEs) could be observed in GF-1 cells 3–5 days post-infection with pancreatic necrosis virus (IPNV), hard clam reovirus (HCRV), eel herpes virus Formosa (EHVF) and GNNV. In addition, abundant GNNV particles were found in the cytoplasm of GNNV-infected GF-1 cells using electron microscopy and nucleic acids of GNNV virus were detected by polymerase chain reaction in the culture medium of GNNV-infected cells after CPE appeared. The experimental results indicated that GF-1 can effectively proliferate fish nodavirus and is a promising tool for studying fish nodavirus.

Introduction

Nervous necrosis virus (NNV), a pathogen found world-wide in many species of hatchery-reared marine fish, has caused mass mortality of fish at the larval or juvenile stages (Yoshikoshi & Inoue 1990; Bruel, Bonami, Pepin & Pichot 1991; Bloch, Gravningen & Larsen 1991; Mori, Nakai, Nagahara, Muroga, Mekuchi & Kanno 1991; Nakai, Mori, Muroga & Mekuchi 1991; Mori, Nakai, Muroga, Arimoto, Mushiake & Furusawa 1992; Comps, Pepin & Bonami 1994; Fukuda, Nguyen, Furuhashi & Nakai 1996; Breton, Grisez, Sweetman & Ollevier 1997; Chi, Lo, Kou, Chang, Peng & Chen 1997; Grotmol, Totland & Kryvl 1997a; Grotmol, Totland, Thorud & Hjeltnes 1997b).

As of the end of 1993, ≈ 159 fish cell lines had been established for isolating and identifying fish viruses (Fryer & Lannan 1994). Most of these cell lines are derived from the tissues of freshwater fish and only 34 cell lines originate from marine fish. Although some fish cell lines, including RTG-2, CHSE-214, BF2, SBL, FHM and EPC, have been tested for susceptibility to fish nodavirus, no cytopathic effect (CPE) has been observed (Bruel *et al.* 1991; Mori *et al.* 1992; Munday, Langdon, Hyatt & Humphrey 1992; Fukuda *et al.* 1996; Delsert, Morin & Comps 1997). In 1996, the SSN-1 cell line was successfully used to isolate sea bass nodavirus (Frerichs, Rodger & Peric 1996), although this cell line has been persistently infected with C-type retrovirus (Frerichs, Morgan, Hart, Skerrow, Roberts & Onions 1991). Therefore, developing a new cell line to replicate fish nodavirus is still an essential task.

The grouper, *Epinephelus coioides* (Hamilton), is an important hatchery fish in Taiwan and severe mortality among groupers has repeatedly occurred

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in recent years. In particular, nodavirus has been isolated from moribund groupers with clinical signs of VNN disease (Chi *et al.* 1997). However, electron microscopy of grouper tissues has revealed other types of virus (Chi 1997). That some viruses are host-specific makes a cell line derived from grouper more appropriate for investigating the viruses isolated from these fish.

In this study, the present authors characterize a new cell line derived from the fin tissue of grouper and report its susceptibility to several aquatic viruses, particularly to a fish nodavirus strain GNNV.

Materials and methods

Primary culture

A specimen of *E. coioides*, weighing 1 kg, was used for the primary culture. The fish was dipped in 5% chlorex for 5 min and then wiped with 70% alcohol. Fin tissue was dissected from the body and washed three times in a washing medium (L15 plus 400 IU mL^{-1} penicillin, 400 μg mL^{-1} streptomycin and 10 μg mL^{-1} fungizone). After washing, the tissue was minced with scissors and placed into 0.25% trypsin solution (0.25% trypsin and 0.2% EDTA in phosphate-buffered saline). The tissue fragments in trypsin solution were slowly agitated with a magnetic stirrer at 4 °C. At 30-min intervals, cells released from the tissue fragment were collected by centrifugation. Then cells were resuspended in complete medium (L15 plus 20% FBS, 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 2.5 μg mL^{-1} fungizone), transferred into 25-mL tissue culture flasks and cultured at 28 °C.

Subculture and maintenance

When the confluent monolayer had formed in primary culture, cells were dislodged from the flask surface by treatment with 0.1% trypsin solution (0.1% tryspin and 0.2% EDTA in PBS) and then transferred into two new flasks after adding fresh L15-20FBS medium. Cells were subcultured at a split ratio of 1:2. For the first 10 subcultures of GF-1 cells, a conditioned medium consisting of 50% old and 50% fresh medium was used. The concentration of FBS in the maintaining L15 medium was 10% for subcultures 11–70, and decreased to 5% after subculture 70.

The GF-1 cell line was propagated for three transfers in antibiotic-free L15–10FCS and tested for the presence of bacteria, fungus and mycoplasma. A Hoechst 33258 stain was used to test the mycoplasma.

Cell storage

The viability of the GF-1 cell line following freezing in liquid nitrogen was tested by removing cells at different subcultures from the flask surface, concentrating by centrifugation, and resuspending in a freezing medium consisting of 10% dimethyl sulphoxide (DMSO) and 90% FBS. A cryotube (Nalge Nunc International, Roskilde, Denmark) containing 5×10^6 cells mL–1 ampule–1 were held at –20 °C for one hour, held at –70 °C overnight and then transferred into liquid nitrogen (–176 °C). After intervals of one month and one year, the ampules were thawed in a 30-°C water bath and the freezing medium removed by centrifugation. Cells were resuspended in L15–10FBS, and the viability of cells was shown by trypan blue staining and the number of cells was counted using a haemocytometer. Finally, the thawed cells were seeded into a 25-mL flask and cultured.

Chromosome number analysis

Chromosome counts were determined for GF-1 cells at subcultures 50 and 80. Semiconfluent and actively growing cells were used. Cells were treated with 0.1 μg mL^{-1} Colcemid (Gibco, Grand Island, NY, USA) for 5 h at 28 °C and dislodged by treatment with 0.1% trypsin solution. After centrifugation at 200 ***g*** for 10 min, the cells were resuspended in a hypotonic solution (eight parts of distilled water and one part PBS) for 30 min and then partially fixed by adding several drops of cold Carnoy fixative (one part glacial acetic acid and three parts 100% methanol). Following centrifugation at 800 ***g*** for 10 min at 4 °C, the supernatant was discarded and the cells were fixed in fresh, cold Carnoy fixative for 20 min. The suspension of fixed cells was dropped onto 76 × 26-mm slides. Following air-drying, the cells were stained with 10% Giemsa (Sigma, St Louis, MO, USA) for 30 min. Finally, the chromosome numbers were observed and counted under an Olympus Vanox microscope.

Plating efficiency

The plating efficiency of GF-1 cells was estimated at

subcultures 50 and 80. Cells were seeded into a 25-mL flask at a density of 100 per flask. Following 15 days of incubation, the medium was removed and cell colonies were fixed with 70% ethanol and stained with 10% Giemsa. The colonies in each flask were then counted using an Olympus IX70 inverted microscope (Olympus Optical Co., Ltd, Tokyo, Japan). For comparison, the plating efficiency of carp fin (CF), black porgy spleen (BPS-1), tilapia (TO-2) and eel kidney (EK) cell lines were also performed using the same tech-nique.

Effects of FBS concentration and temperature on the growth of GF-1 cells

The effects of four concentrations of FBS in a culture medium on GF-1 cell growth were determined at subcultures 50 and 80. Two

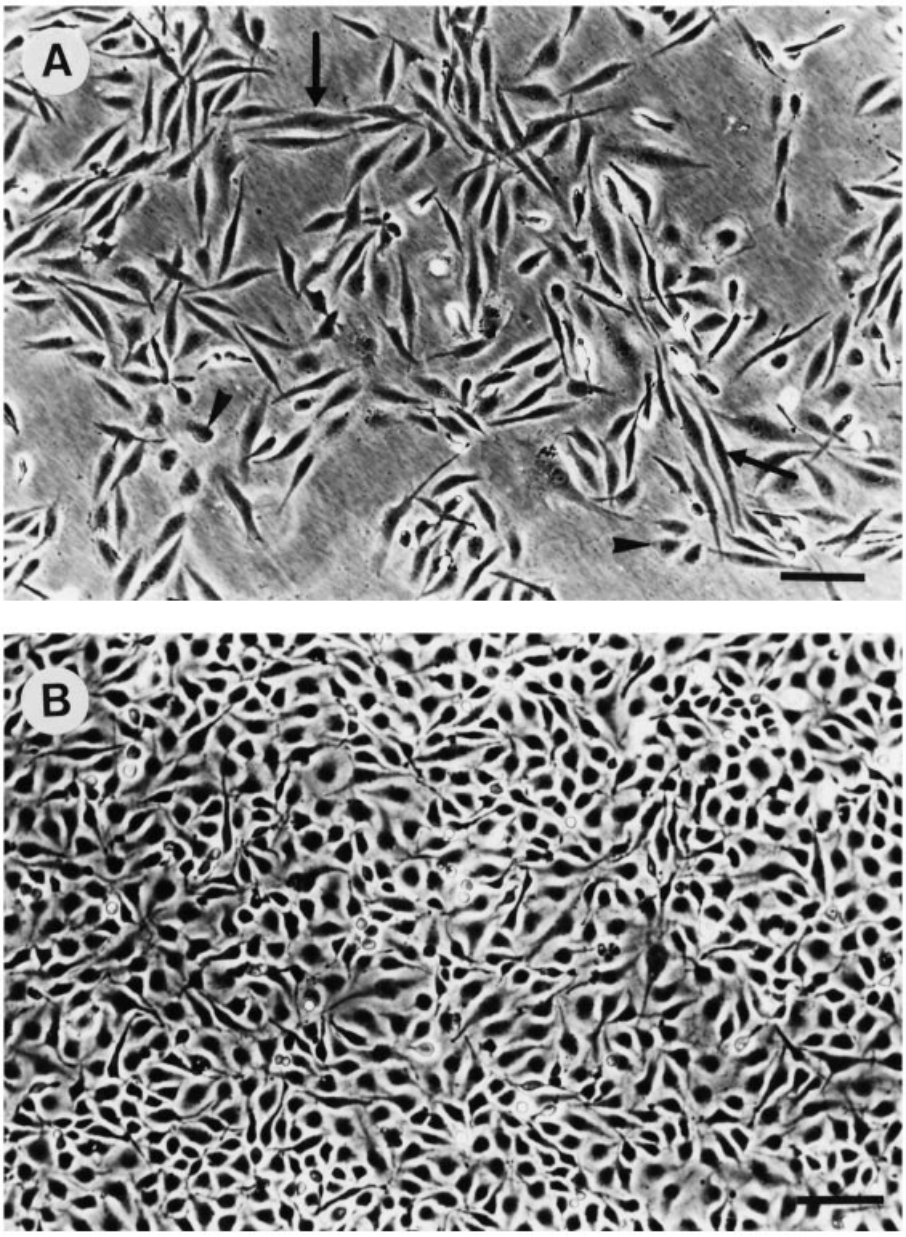

Figure 1 Morphology of GF-1 cells: (a) semiconfluent monolayer and (b) confluent monolayer of GF-1 cells at subculture 80. The arrowhead indicates fibroblast-like cells. The arrows indicate epithelioid cells (bar = 10 μm).

replicates were prepared for each FBS concentration. At selected intervals, two flasks were withdrawn from each concentration of FBS, and the mean number of cells was counted.

To determine how temperature affects the growth of GF-1 cells at subculture 80, replicated cell cultures in 25-mL flasks containing L15–10 FCS were incubated at 18, 28 and 35 °C. The mean number of GF-1 cells from two replicated flasks at each temperature were counted at selected intervals.

Susceptibility of GF-1 cells to aquatic viruses

Infectious pancreatic necrosis virus (IPNV, strains AB, SP, VR299 and EVE), hard clam reovirus (HC RV), eel herpes virus Formosa (EHVF; Shih, Lu & Chen 1993), and grouper nervous necrosis virus (GNNV MT9410; Chi *et al.* 1997) were used to infect GF-1 cells at subculture 80. The susceptibility to GNNV was also examined in the GF-1 cell line at subculture 50 and in the BGF-1 cell line, which was derived from the fin of banded grouper, *Epinephelus awora* (Temminck & Schlegel), (Chen & Kou 1988).

A monolayer of GF-1 cells was inoculated with 0.5 mL GNNV with a titre of 10^3 TCID 50 0.1 mL^{-1}. After a 30-min adsorption period, the cells were washed three times by PBS, and 5 m L L15–2FBS was added to each flask. The flasks were then incubated separately at 20 and 28 °C. Supernatants of culture cells were collected and titrated 6 days post-infection.

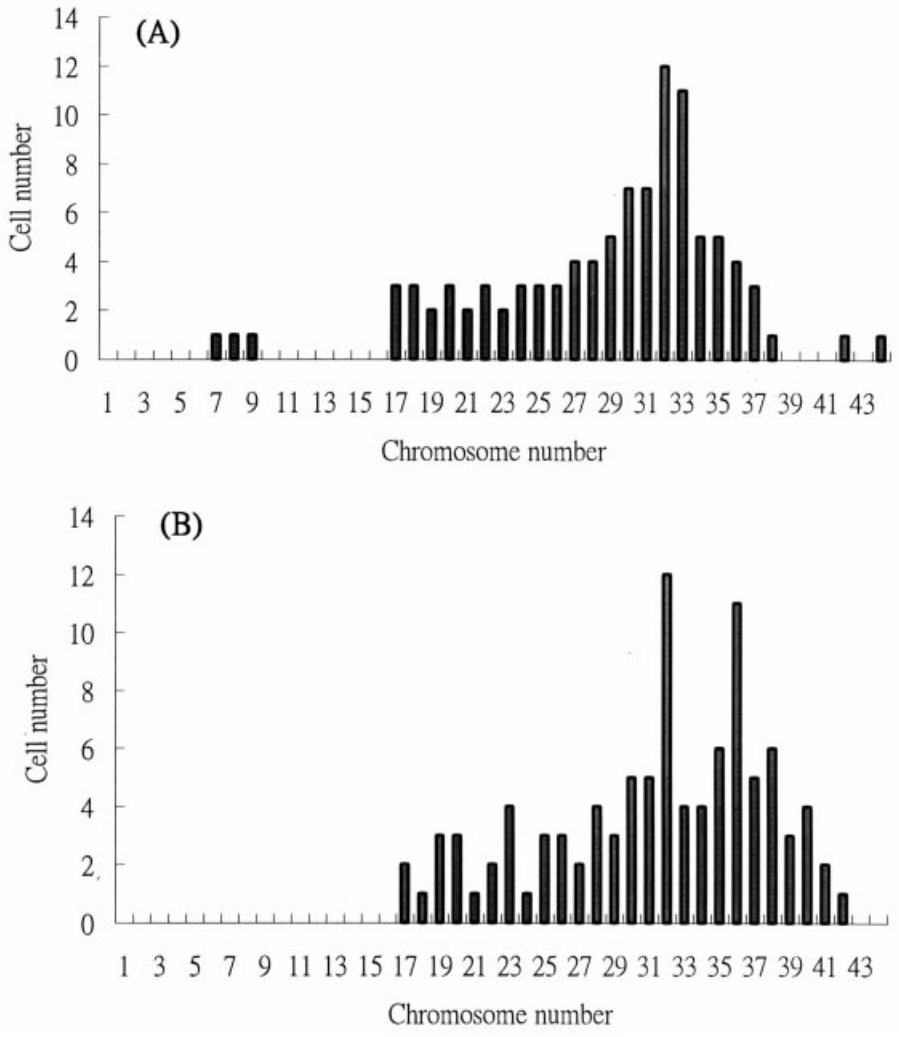

Figure 2 Chromosome number distribution of GF-1 cells at (a) subculture 50 and (b) subculture 80 (n = 100 cells).

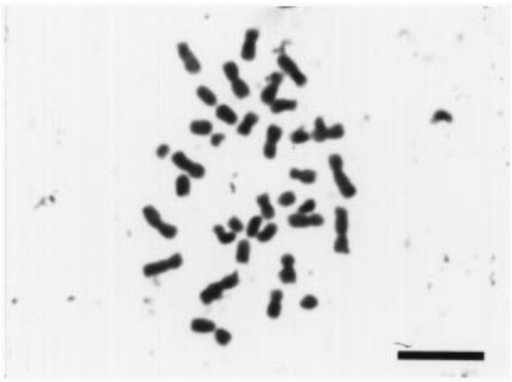

Figure 3 Metaphase chromosomes from a GF-1 cell (bar = 1 μm).

Identification of GNNV replication in GF-1 cells by polymerase chain reaction (PCR) amplification

To reconfirm that GF-1 cells at subculture 80 can proliferate GNNV, the supernatant was taken from the GNNV-infected cells after CPE appeared, and viral RNA extracted from the supernatant using a Rneasy mini kit (QIAGEN Companie, Hilden, Germany). For reverse transcription, extracted viral RNA was incubated at 42 °C for 30 min in 40 μL 2.5 × PCR buffer (25 mM Tris-HCl, pH 8.8, 3.75 mM $MgCl_2$, 125 mM KCl, 0.25% Triton X-100) containing 2 units of MMLV reverse transcriptase (Promega Corp. WI), 0.4 U RNsin (Promega Corp. WI), 0.25 mM dNTP and 0.5 μM reverse primer. Following cDNA synthesis, 40 μL of the cDNA mixture was diluted 2.5-fold with diethyl pyrocarbonate (DEPC)-treated H_2O containing 0.025 units DNA polymerase (Boehringer Mannheim Biochemicals, Mannheim, Germany), 0.1 mM dNTP and 0.5 μM forward primer, and incubated in an automatic thermal cycler (TouchDownTM Thermal Cycler, Hybaid Limited, TM, UK). This experiment used reverse primer R3 (5′ CGAGTCAACACGGGTGAAGA 3′) and forward primer F2 (5′ CGTGTCAGT-CATGTGTCGCT 3′), designed by Nishizawa, Nakai, Furusawa & Muroga (1994). The target region for the primer set (F2, R3) is T4 (426 bp).

Electron microscopy for detection of GNNV in GF-1 cells

The GNNV-infected GF-1 cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 and post-fixed in 1% osmium tetroxide. Ultrathin sections were stained with uranyl acetate-lead citrate and examined under a Hitachi H-600 A electron microscope (Hitachi, Corp., Tokyo, Japan).

Results

Primary culture and subculture of GF-1 cells

Monolayers of cells in the primary culture formed approximately 2 weeks after implantation. Fibroblast-like cells and epithelioid cells were both found in the cell population (Fig. 1). The GF-1 cells have been successfully subcultured more than 160 times since 1995, subsequently becoming a continuous cell line.

The GF-1 cells were subcultured at 9-day intervals in L15-20FCS during the first 20 subcultures, at 5-day intervals in L15-10FCS during subcultures 21–70, and at 3-day intervals in L15-5FCS after subculture 71. Contact inhibition of GF-1 cells, although obvious in the cells before subculture 50, gradually decreased between subcultures 51–80.

The GF-1 cells at subculture 80 were frozen in liquid nitrogen and the viability was tested after one

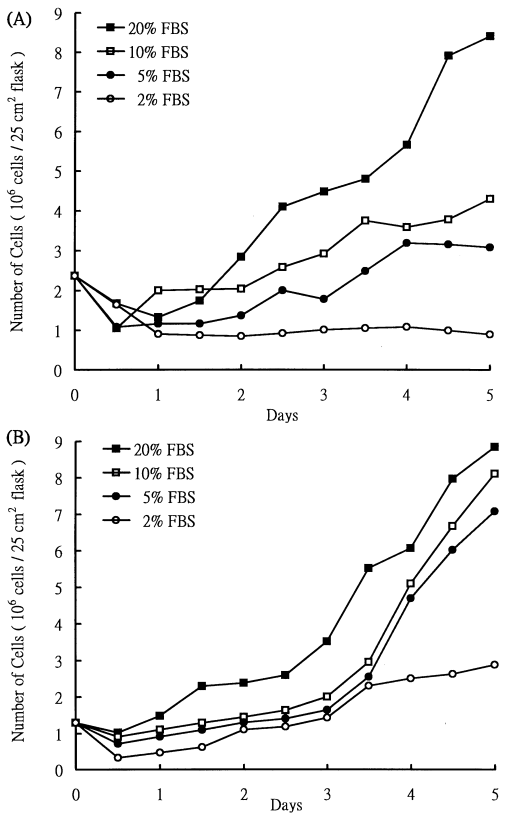

Figure 4 Effect of FBS on the growth rate of GF-1 cells at (a) subculture 50 and (b) subculture 80.

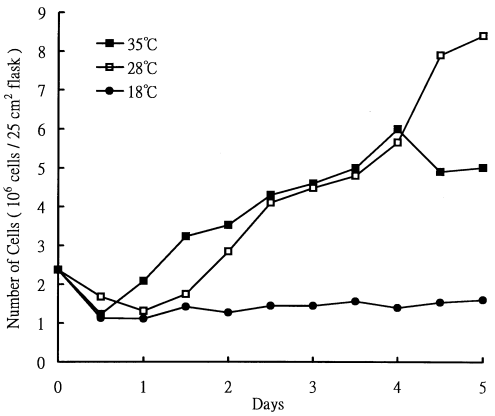

Figure 5 Temperature effect on growth rate of GF-1 cells at subculture 80.

year. The survival rate of GF-1 cells was 73% and the cells grew readily when incubated at 28 °C in L15–5FCS.

Chromosome number

Chromosome numbers of GF-1 cells at subculture 50 distributed from 7 to 44, with a mode number of 32 (Fig. 2a). The GF-1 cells at subculture 80 showed chromosome distribution from 17 to 42, and had a bimodal distribution with modes at 32 and 36 (Fig. 2b). Both micro- and macro-chromosomes were found in metaphase-arrested cells (Fig. 3).

Plating efficiency

The plating efficiency of GF-1 cells seeded at a density of 100 cells $flask^{-1}$ was 21% at subculture 50 and increased to 80% at subculture 80. In comparison, the plating efficiencies of CF, BPS, To–2 and EK cell lines seeded at a density of 100 cells $flask^{-1}$ were 22%, 13%, 48% and 63%, respectively. In addition, the increase in plating efficiency in GF-1 cells indicated transformation during subcultures 50–80.

Effect of FBS concentration and temperature on the growth curve of GF-1 cells

Figure 4 illustrates how FBS concentration affects the growth of GF-1 cells at subcultures 50 and 80. The growth rates of GF-1 cells at subculture 80 in medium containing 2%, 5% and 10% FBS are higher than that of GF-1 cells at subculture 50 with the same FBS concentration (Fig. 4b). These results suggest that the requirement of FBS for cell growth decreased at subculture 80, and that further transformation occurred between subcultures 50 and 80.

Figure 5 shows the temperature effect on GF-1 cells at subculture 80. Although cells grew well at 28 and 35 °C, the growth rate of cells cultured at 35 °C began to decrease after day 4. The growth of GF-1 cells was limited at 18 °C.

Virus susceptibility

Table 1 summarizes the virus susceptibility of GF-1 cells to IPNV (AB, SP, VR299 and EVE strains), HCRV, EHVF and GNNV. Typical CPE was observed in GF-1 cells infected with these viruses (Fig. 6). The CPE of IPNV strains and HCRV appeared only at 20 °C; CPE of EHVF appeared at both 20 and 28 °C, while CPE of GNNV only appeared at 28 °C (Table 1). Notably, yields of these viruses in GF-1 cells at subculture 80 were extremely high (Table 1). Typical CPE of GNNV began at day 3 post-infection, and CPE developed

Table 1 Susceptibility of GF-1 cells at subculture 80 to different viral isolates*

		Cytopathic effect		Virus yield mL^{-1} ($TCID_{50}$ mL^{-1})	
Virus	Initial viral inoculum ($TCID_{50}$ mL^{-1})	28 °C	20 °C	28 °C	20 °C
IPNV:					
AB	10^3	–	+	ND	$10^{9.5}$
SP	10^3	–	+	ND	$10^{10.8}$
VR299	10^3	–	+	ND	$10^{9.8}$
EVE	10^3	–	+	ND	$10^{9.6}$
HCRV	10^3	–	+	ND	$10^{11.0}$
EHVF	10^3	+	+	$10^{8.1}$	$10^{7.0}$
GNNV	10^3	+	–	$10^{8.3}$	ND

*ND: not done; (+) cytopathic effect was observed.

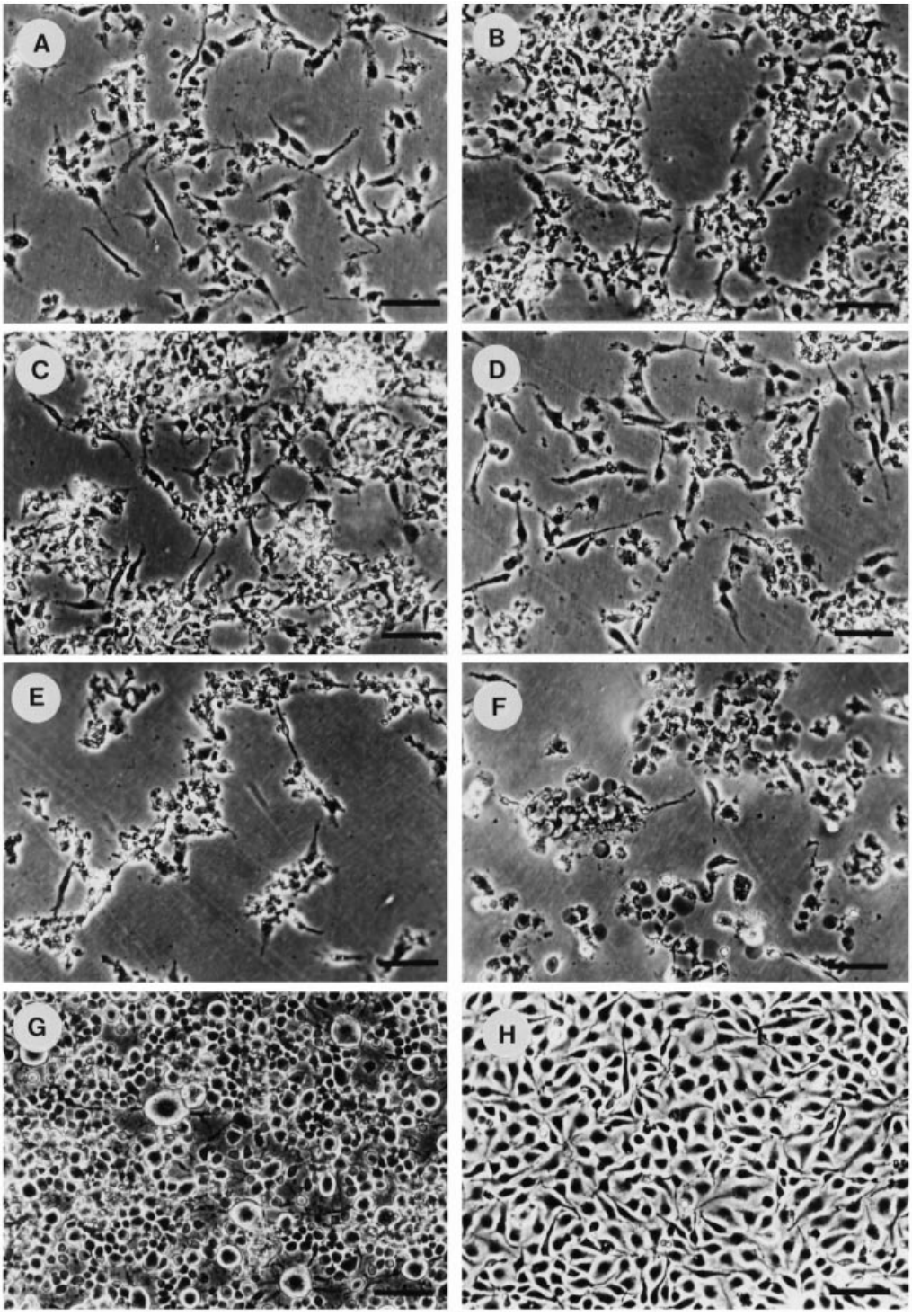

Figure 6 The CPE of GF-1 cells at subculture 80 after infection by (a) IPNV AB strain, (b) IPNV SP strain, (c) IPNV VR299 strain, (d) IPNV EVE strain, (e) HCRV, (f) GNNV MT9410 strain, (g) HEVF and (h) uninfected GF-1 cells.

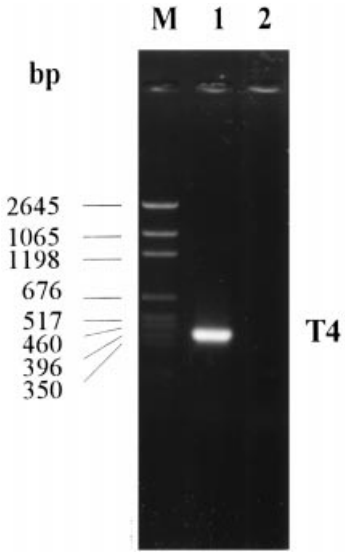

Figure 7 Agarose gel electrophoresis of the product by RT-PCR amplification using a primer set (F2, R3) specific to the target region T4 of fish nodavirus SJNNV. Lanes: (1) PCR product from GNNV-infected GF-1 cells; and (2) PCR product from non-infected GF-1 cells; (M) pGEM marker.

initially as areas of rounded, granular, refractile cells and then spread to the entire cell sheet. Finally, the cells degenerated and detached (Fig. 6f).

No CPE was found in GNNV-infected GF-1 cells at subculture 50. The BGF-1 cell line (Chen & Kou 1988) did not show any CPE after infection with GNNV.

Identification of GNNV replication in GF-1 cells by PCR amplification

The target fragment T4 of fish nodavirus specific primer set (F2, R3) was detected after PCR amplification of extracted nucleic acid from the supernatant of GNNV-infected GF-1 cells (Fig. 7). This indicates that GNNV replicated in the GF-1 cells and was then released into the supernatant of culture cells.

Electron microscopy of GNNV in GF-1 cells

Electron microscopy of GNNV-infected GF-1 cells revealed that many non-enveloped viral particles

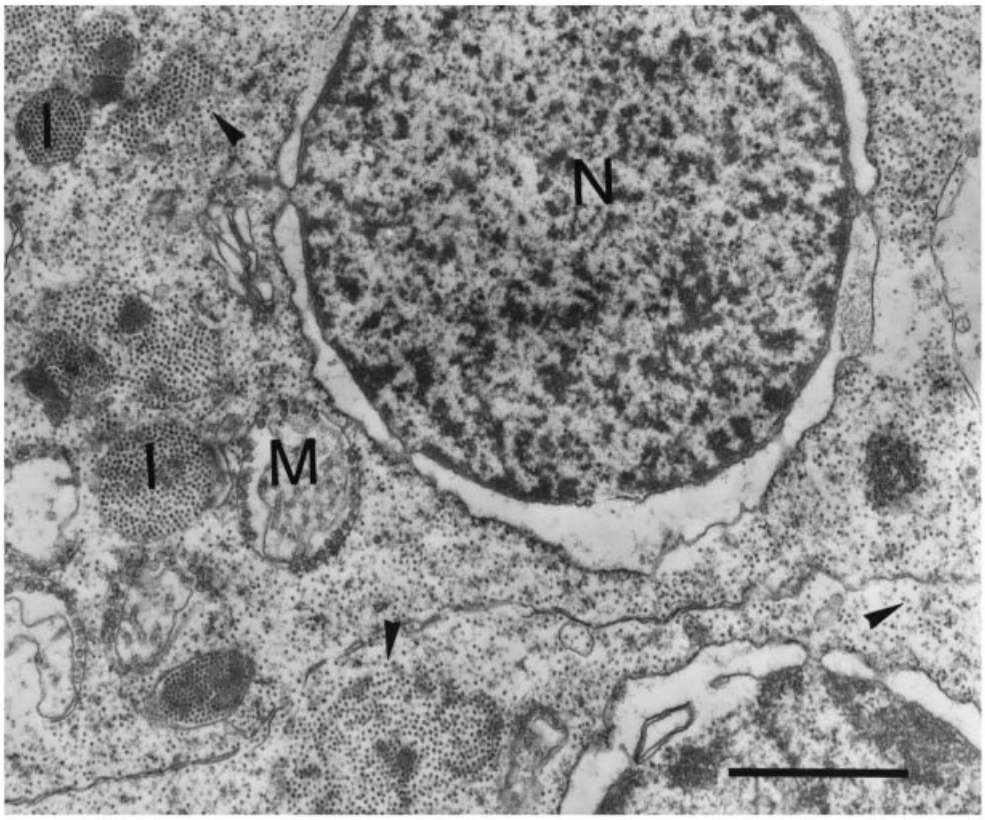

Figure 8 Electron micrograph of GNNV-infected GF-1 cells: (I) inclusion body filled with viral particles; (M) mitochondria; and (N) nucleus. The arrowhead indicates non-enveloped viral particles (bar = 1 μm).

were enclosed in the inclusion bodies or randomly distributed in the cytoplasm (Fig. 8), confirming that GNNV can replicate in GF-1 cells.

Discussion

In primary culture of GF-1 cells, epithelial cells and firobroblast-like cells coexisted. However, in subsequent subcultures, the fibroblast-like cells proliferated more rapidly than the epithelial cells and ultimately predominated. Many serum factors derived from platelets have a strong mitogenic effect on fibroblasts and also tend to inhibit epithelial proliferation, subsequently causing fibroblasts to overgrow in subcultures (Freshney 1994).

In comparison to GF-1 cells at subculture 50, those at subculture 80 displayed lower serum dependence, higher plating efficiency and reduced contact inhibition. These changes suggest that spontaneous transformation occurred between subcultures 50 and 80. The fact that many properties associated with transformation *in vitro* were reported to be a consequence of cell surface modifications (Freshney 1994) suggests the possibility that the *de novo* susceptibility of subculture 80 to GNNV may be attributed to the generation of a new receptor to GNNV on the surface of the GF-1 cells.

Although originating from fin tissue of banded grouper and composed of epithelial cells, the BGF-1 cell line (Chen & Kou 1988) did not show CPE after GNNV infection. Therefore, the BGF-1 cell line has different properties from those of the GF-1 cell line.

Previous investigations have indicated that viral nervous necrosis disease (VNN disease) of groupers occurs in summer when water temperature ranges from 28 to 32 °C (Fukuda *et al.* 1996; Chi *et al.* 1997). This suggests that such high temperatures are necessary for the replication of grouper nervous necrosis virus. The GF-1 cells grow very well at 28 °C and are susceptible to GNNV, as indicated by the detection of ENNV nucleic acids in the supernatant of infected GF-1 cells and the observation of abundant GNNV particles in the cytoplasm of infected cells, confirming the potential of the GF-1 cell line as an effective tool for the study of fish nodavirus.

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