The Mechanism of Interference with Fish Viral Infection in the RTG-2 Cell Line

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The influence of cell density on viral infectivity titers and interferon production were studied by a plaque and end-point dilution method. The RTG-2 cell line was infected with eel virus European (EVE), infectious pancreatic necrosis virus (IPNV) and infectious hematopoietic necrosis virus (IHNV). The confluent condition of RTG-2 cells was classified into two phases according to the cell density, viz. loosely confluent (6.0 x 10⁴ cells/cm²) and tightly confluent cell sheets (1.2 x 10⁵ cells/cm²). Viral infectivity titers were highest in loosely confluent cell sheets. Virus titers decreased by 50-70% in a plaque method and 90-99% by end-point dilution in tightly confluent cell sheets. Conversely, more interferon was produced in tightly rather than loosely confluent cell sheets. We may, therefore, conclude that interferon seems to be a factor contributing to the significant reduction of viral infectivity titers in tightly confluent cell sheets.

Introduction

The study of fish virology has made rapid progress since fish cell lines were established (Wolf and Quiamby, 1962; Gravell and Malsberger, 1969; Wolf and Mann, 1980). Quantitative analysis of fish viruses has been carried out by a 50% tissue culture infectious dose (TCID₅₀) method, a plaque (PFU) method and an immunofluorescent cell assay (IFC). However, studies concerning the relationships between cell density and viral infectivity titer with fish viruses have not been done, although it was reported that the age of RTG-2 cell cultures influenced the susceptibility of the cells (Tu et al., 1974).

In this paper we report the influence of cell density on viral infectivity titer and interferon production by a plaque method and a microtiter system.

Materials and Methods

Cell Culture

RTG-2 cells (Wolf and Quiamby, 1962) in 176th to 186th passages were used in this study. The stocks of RTG-2 cells were grown at 20°C in Eagle's minimum essential medium (Nissui Seiyaku Co.) with 10% fetal bovine serum (GIBCO Lab.), 100 I.U. penicillin and 100 μg streptomycin/ml buffered at pH 7.6 to 7.8 with 16 mM tris-HCl (MEM-10). RTG-2 cells for virus titrations were prepared from cell sheets where the increase of cell density had stopped.

Virus

Viruses used in this study were eel virus European (EVE) (Sano et al., 1981; Okamoto et al., 1983), infectious hematopoietic necrosis virus (IHNV) (Sano et al., 1977) and ATCC VR 299 strain of infectious pancreatic necrosis (IPNV) provided by Dr. K. Wolf, National Fish Health Research Laboratory, U.S.A. These viruses were plaque purified and have been passed in RTG-2 cells thirteen, ten and sixteen times, respectively. The stock viruses were prepared at 18°C by the method of Sano et al. (1981).

Virus Assay

Virus titrations were carried out at 18°C using the end-point dilution method (TCID₅₀).
Quantitation of Cell Growth

Cells at a density of $6.0 \times 10^4$ per cm$^2$ were suspended in 5.0 ml of MEM-10, inoculated into each of forty tissue culture petri dishes 5.0 cm in diameter (Falcon Plastics) and incubated at 18°C. Five petri dishes per day were used for the purpose of counting the cell numbers. Cell counts were made every day for 7 days. The cell sheets were dispersed with 2.0 ml of dispersant containing 0.02% EDTA and 0.1% trypsin in PBS (without Mg$^{2+}$ and Ca$^{2+}$). They were then added to equal parts of MEM-10 to stop the activity of dispersant. Cell counts were made with cells in suspension, using a red blood cell counting chamber (Thoma).

Viral Infectivity Titer and Cell Density

Cells (6.0 $\times$ 10$^4$ per cm$^2$) were inoculated into 5.0 cm diameter tissue culture petri dishes and microtiter plates (Falcon Plastics) with 5.0 ml and 0.1 ml of MEM-10, respectively, and incubated at 18°C. Viral infectivity titers were determined in 0–6-day old cell sheets by the end-point dilution method and in 1–7-day old cell sheets by the plaque method, using the stock EVE and IHN viruses.

Interferon Assay

Interferon was assayed using the modified neutral red dye uptake method of FINTER (1969). A modification of the method described by de SENA and Rio (1975) was employed to assay interferon produced by RTG-2 cells. The assay was performed using RTG-2 cell monolayers in 16 $\times$ 125 mm culture tubes. Cells (2.6 $\times$ 10$^6$) suspended in 1.0 ml of MEM-10 were inoculated into tubes and incubated at 20°C for 24 h. The monolayers were approximately 90% confluent after this period. RTG-2 cell interferon preparations were serially diluted (0.2 log per dilution) in MEM-5 (pH 7.5). Three tubes were used at each dilution and each tube received 1.0 ml of interferon. Monolayers with interferon were incubated at 20°C for 24 h and washed twice with Hanks' BSS. Each monolayer received $10^{9.0}$ TCID$_{50}$ per ml of IPNV (strain VR 299). The tubes were then placed at 20°C for 42–72 h until cytopathic effect (CPE) developed. Dye solution (0.4 ml) containing 0.02% neutral red and 0.85% NaCl in distilled water was then added to each assay tube. The tubes were placed in the dark at 20°C for 1 h. Residual neutral red was then removed by washing the monolayers twice with physiological saline. The dye was extracted from the cells in each tube with 3.0 ml of a mixture containing equal parts of absolute ethanol and 0.1 M Na$_2$HPO$_4$ (pH 4.3). Absorbance of the extracted dye was determined at a wave length of 540 nm employing a Hitachi 101 spectrophotometer.

Interferon Production and Cell Density

Cells (2 $\times$ 10$^4$ per cm$^2$) suspended in 15 ml of MEM-10 were inoculated into glass bottles (approximately 60 cm$^2$ growth area) and incubated at 18°C. Cell monolayers on 2, 6, 10 and 14 days post-inoculation were used to produce crude interferon. The RTG-2 crude interferon had previously been shown to be interferon (SANO and NAGAKURA, 1982). To produce RTG-2 crude interferon, the growth medium was decanted and the cells washed twice with Hanks' BSS. Each bottle was then inoculated with 3.0 ml of Hanks' BSS containing $10^{6.0}$ TCID$_{50}$ of IHNV. The bottles were placed at 18°C for 1 h. The monolayers were then gently rinsed twice with Hanks' BSS and 5.0 ml of MEM-10 were added to the culture bottles. The cells were incubated at 18°C for 72 h. Overlay fluids were removed and clarified by centrifugation at 5,000 $\times$ g for 20 min. The supernatants were brought to pH 2.0 by the addition of concentrated HCl and placed at 4°C for 48 h. The pH of the supernatants was adjusted to 7.4 by the addition of saturated NaOH and interferon activity in the supernatants was assayed by the dye uptake method described.

Results

Quantitation of Cell Growth

The growth curve of RTG-2 cells in MEM-10 at 18°C shows that there were approximately
6.0 × 10^{4} cells per cm² in 0–2-day old cell sheets (Fig. 1). On 3-day old cell sheets, the density reached about 1.2 × 10^{5} cells per cm² and after that cell density increased very little. Cells at a density of 6.0 × 10^{4} per cm² made loosely confluent cell sheets and 1.2 × 10^{5} cells per cm² formed a tightly confluent mass (Fig. 2).

**Viral Infectivity Titer and Cell Density**

Titrations of EVE and IHNV by both the plaque and end-point dilution methods showed that 0–2-day old cell sheets (loosely confluent) produced the highest titers (Fig. 3). Titers of EVE were 10^{7.8} TCID_{50}/ml and 1.9 × 10^{6} pfu/ml and for IHNV they were 10^{8.3} TCID_{50}/ml and 3.7 × 10^{6} pfu/ml. Viral infectivity titers were markedly decreased on 3-day old cell sheets showing a tightly confluent condition. The decrements were 90\% for EVE and 99\% for IHNV by end-point dilution, and 70 and 50\%, respectively, by the plaque method.

Cell sheets older than 3 days (tightly confluent) produced the same titer as found on 3-day old cell sheets by end-point dilution whereas they slightly less by the plaque method. When the stock EVE and IHNV were titrated on fresh cell sheets (6.0 × 10^{4} cells per cm², loosely confluent) after completion of this study, the same viral titers as those found on 0–2-day old cell sheets were obtained by both the end-point dilution and plaque methods.

**Interferon Production and Cell Density**

Infectious hematopoietic necrosis virus was added on those days at which the cell monolayers were 2, 6, 10 and 14-day old. Two-day old cell monolayers produced no interferon (Fig. 4), 32 DU_{50}/ml of interferon activity was produced by 6-day old cell monolayers, and activities of 105 DU_{50}/ml and 115 DU_{50}/ml
Fig. 3. The relationship between viral infectivity titer and cell density. Viral infectivity titer shown are expressed in percentage of which the measures were divided by the highest titer. The marks of ○ and ● show IHNV and EVE, respectively. Left is the titer obtained by a plaque method. Right is the titer obtained by the end-point dilution method in a microtiter system.

Discussion

PLUMB and WOLF (1970) reported the growth rate of RTG-2 cells and according to their study, it took 2 days for RTG-2 cells to double in density at 20°C and 3 days at 15°C. Our results showed the doubling time of RTG-2 cells was 3 days at 18°C.

It has been known that viral infectivity titers are influenced by the age of cells and the confluency of cell sheets. It is required that cell sheets are in a confluent condition for plaquing. Therefore, in our study, the sensitivity of cells was tested after RTG-2 cells formed confluent cell monolayers. Cell density of approximately $6.0 \times 10^4$ per cm$^2$ was maintained for two days after seeding at same cell density. The cell density reached $1.2 \times 10^5$ cells per cm$^2$ on the 3rd day after seeding. Viral infectivity titers (Fig. 3) showed the highest point while the cell density remained approximately $6.0 \times 10^4$ cells per cm$^2$ (0-2-day old cell sheets). However, when the cell density reached $1.2 \times 10^5$ cells per cm$^2$, the viral infectivity titers decreased by 50-70% in the plaque method and 90-99% by end-point dilution. Although the cell sheets were judged confluent by light microscopy at both cell densities, $6.0 \times 10^4$ cells per cm$^2$ and $1.2 \times 10^5$ cells per cm$^2$ (Fig. 2), the viral infectivity titers were significantly different as is evident from the above. Therefore, confluent cell sheets can be classified into two phases. The former was called loosely confluent and the latter tightly confluent. Few changes of viral infectivity titer were recorded in the cells after reaching a tightly confluent condition, however, a remarkable change of RTC-2 cell susceptibility occurred, when the cell sheets grew from loose to tight confluency. It was reported...
that RTG-2 cells produce interferon (de SENA and RIO, 1975; SANO and NAGAKURA, 1982) and in our studies interferon production was greater in tight confluent cells (Fig. 4). From this, we conclude that interferon is a factor contributing to the noticeable reduction of viral infectivity titers in tightly confluent cells.

Tu et al. (1974) reported that 1 or 2-day old RTG-2 cells were less susceptible to IPN virus infection than older cells. In our study, however, the results showed that 1 or 2-day old RTG-2 cells were more susceptible than the older ones. Although our results differ from those of Tu et al., it is difficult to compare these since there were differences in methodology. In their study, they used an immunofluorescent antibody method to quantify virus infected cells while we used a plaque and end-point dilution methods for viral titrations. In addition, the cell density and methods for preparing cells were not reported in their study.

MCLAREN (1970) reported interesting data concerning the effect of aging of L-cells on their sensitivity to exogenous interferon and to infection with Semliki Forest Virus (SFV). Viral infectivity titers of SFV gradually increased in 1-day old to 5-day old cells with both end-point dilution and immunofluorescent cell counts. The most susceptible cells were 5-day old cells and the viral infectivity titer at that time was $5.0 \times 10^6$ TCID$_{50}$/ml and $>1.1 \times 10^4$ FD$_{50}$/ml, respectively for each method. However, 7-day old cells rapidly became resistant to SFV with a viral infectivity titer of $5.0 \times 10^4$ TCID$_{50}$/ml and $2.4 \times 10^4$ FD$_{50}$/ml, respectively. This was in spite of the fact that viable cell counts were twice that of the 5-day old cells. Furthermore, it was reported in the same paper that 7-day cells produced the largest amount of interferon and were the most sensitive to exogenous interferon. MCLAREN (1970) did not explain why 7-day old cells became suddenly resistant to SFV. The data reported by MCLAREN (1970) has similarity to ours except he did not document the confluency of the 5 and 7-day old cells. We believe that the series of phenomena reported by him might well be explained by our observations that remarkable changes in cell susceptibility to viruses and interferon production are related to different cell conditions ranging from loosely to tightly confluent.

The decrease of viral infectivity titers by end-point dilution was greater than in the plaque method (Fig. 3). KATO and EGGERS (1969) reported that the enhanced capacity of chick embryo cells to produce interferon appears to be mediated by a factor produced in aging cultures and released into the medium. Although no one has reported whether or not such a factor is produced in aging cultures of RTG-2 cells, the authors suggested that the factor might account for the lower viral infectivity titers by end-point dilution because the aged medium was removed in the plaquing method, while it remained when end-point dilution methods were used.

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References


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魚類ウイルスに対する干渉メカニズムと RTG-2 細胞の繁茂状態

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ウイルス感染力価とインターフェロン産生能に及ぼす細胞密度の影響を明らかにし、ウイルスに対する細胞の干渉メカニズムの解明を試みた。供試細胞は RTG-2 細胞であり、供試ウイルスは EVE, IPNV, IHNV である。RTG-2 細胞の繁茂状態を loosely confluent (6.0×10^4 cells/cm^2) と tightly confluent (1.2×10^5 cells/cm^2) に分けた。ウイルス感染力価は細胞の繁茂状態が前者のときに高かった。後者の状態では前者の状態に比べ、その力価はプラック法で 50～70%、マイクロタイター法で 90～99% 低かった。一方、インターフェロン産生量は細胞の繁茂状態が tightly confluent で loosely confluent より多くなった。故に、細胞の tightly confluent 状態でウイルス感染力価が低く測定される原因は、同細胞状態でのインターフェロン産生能の高まりにあると推察した。