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Susceptibility of a continuous murine cell line (GRX) to viral infection

Suscetibilidade de uma linhagem celular murina contínua (GRX) à infecção viral

Susceptibilidad de un linaje celular murino continuo (GRX) a la infección viral

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ABSTRACT

The ability of a murine cell line (GRX) to support viral replication was evaluated. GRX cell cultures were infected with different DNA or RNA viruses. It was observed that the GRX cell line is susceptible to the replication of *Herpes simplex virus* types 1 and 2 (HSV-1 and HSV-2), *Mayaro virus* (MAY), *Sindbis virus* (SIN), and *West equine encephalitis virus* (WEE), and can be used as substrate for viral replication studies. Viral replication induced cytopathic effect (CPE) 24-48 h post-infection. The GRX cells yielded infectious virus titers between $10^{2.4}$ $TCID_{50}$ (Tissue Culture Infectious $Dose_{50}$) /25 µL and $10^{5.4}$ $TCID_{50}$/25 µL in the first viral passage. These results demonstrate that GRX cells efficiently sustain viral replication and therefore can be used as a valuable tool in the virology laboratory.

Keywords: Cell culture technique; Hepatic Stellate Cells (GRX); Virus replication.

During the last two decades, the application of molecular and serological methodologies has had a major impact on the detection of novel viruses, yet viral isolation remains the gold standard method for the identification and characterization of their biological and biochemical properties. Cell culture is still the most common method for propagation of viruses. Cell culture methods are also used for vaccine production and biochemical and molecular biological studies of viral replication[1]. In 1985, GRX, a continuous cell line representative of hepatic stellate cells, was established from fibro-granulomatous lesions induced in mouse liver by schistosomal infection[2], and its biological and biochemical characteristics were determined[3,4,5]. It is a highly proliferative cell line showing fibroblastic morphology with a stellate, polygonal or elongated shape. When the monolayers are confluent, the cells gather in a distinct "hills and valleys" pattern of growth[2]. The ability of the GRX cell line to support viral replication has never been shown; therefore, the primary goal of this work was to demonstrate the usefulness of the GRX cell line for propagation of selected viruses.

The GRX cells were obtained from the Banco de Células do Estado do Rio de Janeiro, Rio de Janeiro, Brazil. The African green monkey kidney (Vero), rhesus monkey kidney (MA-104), and human epithelial (HEp-2) cell lines were obtained from stocks used for routine viral isolation in the Virology Department of the Microbiology Institute, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil. All cell lines were cultured in Minimum Essential

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Medium (MEM) supplemented with 5% (HEp-2 cells) or 10% (Vero, MA-104, and GRX cells) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 50 μg/mL gentamicin, 2.5 μg/mL fungizone, sodium bicarbonate at 0.25%, and 10 mM HEPES. Cell cultures were incubated at 37° C with 5% CO_2.

The virus strains used in this paper were obtained from the reference collection of the Virology Department of the Microbiology Institute, Universidade Federal do Rio de Janeiro. *Herpes simplex virus* type 1 (HSV-1) and type 2 (HSV-2), *Sindbis virus* (SIN), *Mayaro virus* (MAY), and *West equine encephalitis virus* (WEE) were maintained in Vero cells; *Adenovirus* (AdV) serotypes 2, 19, 40, and 41 were maintained in HEp-2 cells; and *Rotavirus* (RV) strain SA-11 was maintained in MA-104 cells.

To propagate the viruses, cell monolayers were prepared in 48-well plates; each viral suspension was inoculated in triplicate. The inoculated plates were incubated for 1 h at 37° C. Subsequently, the inocula were replaced with fresh MEM without FBS (maintenance medium). For rotavirus, trypsin was added to the inoculum at a final concentration of 10 μg/mL, followed by incubation at 37° C for 30 min. Then the inoculum was added to the cell monolayer with maintenance medium at a final trypsin concentration of 5 μg/mL. All infected cell cultures were incubated at 37° C with 5% CO_2. The cells were monitored daily for the development of viral cytopathic effect (CPE) using an inverted microscope. When approximately 75% of the cells in the culture showed CPE or after seven days of incubation in the absence of CPE, the infected cell cultures were subjected to three freeze-thaw cycles. Afterwards, the mixtures of cell lysates and supernatants were collected and stored at -70° C for future use. Each virus strain was subjected to three serial passages in GRX cells. The mixtures of supernatant and cell lysate (viral suspension) from each of the three passages were subjected to titration by the endpoint dilution method to quantify viral infectivity. Logarithmic dilutions of 25 μL of the viral suspension were inoculated onto confluent monolayers in 96-well plates (six wells/dilution). Titers were calculated according to the Reed and Muench method and expressed in $\log_{10}$ $TCID_{50}$ (Tissue Culture Infectious $Dose_{50}$)/25 μL[6].

In 24-well plates, GRX cells at a density of 4.2 x 10^5 cells/mL and Vero cells at a density of 1.2 x 10^6 cells/mL were plated in MEM supplemented with 10% FBS and infected with 100 μL of viral suspension (MAY, SIN or WEE) at a multiplicity of infection (MOI) of 1.0, 0.5, and 0.1. The infected cell cultures were incubated at 37° C with 5% CO_2 for 48 h. After that period, the viral suspension was collected and titrated as described above. All experiments were performed in triplicate.

Statistical analysis was performed using analysis of variance (ANOVA) to determine the least significant difference at $p = 0.05$. The analysis was performed with Minitab® for Windows, Release 14.0 (Minitab Inc., State College, PA, USA). The term "significance" (statistically significant) in the text means $p \leq 0.05$.

GRX cells were susceptible to infection with HSV-1, HSV-2, SIN, and WEE, producing visible CPE in 48 h. MAY induced CPE in 24 h. In contrast, GRX cells did not support detectable viral replication when inoculated with AdV or RV (Table 1). The CPE observed for MAY, SIN, and WEE on GRX cells was cell pyknosis followed by cytoplasm fragmentation and detachment from the monolayer, which was similar to the CPE observed in Vero cells. For HSV-1 and HSV-2, the CPE observed was rounding and refringence of the cells (Figures 1 and 2).

Table 1– Infection of GRX cells by different RNA or DNA viruses

Virus strain†	Virus titers‡		
	1*	2	3
RNA virus			
MAY	4.84	5.0	7.4
SIN	5.0	6.0	6.5
WEE	5.4	5.5	6.6
RV	-§	—	—
DNA virus			
HSV-1	2.75	3.25	3.5
HSV-2	2.6	2.4	3.4
AdV2	—	—	—
AdV19	—	—	—
AdV40	—	—	—
AdV41	—	—	—

†MAY- *Mayaro virus*, SIN - *Sindbis virus*, WEE - *West equine encephalitis virus*, RV - *Rotavirus*, HSV - *Herpes simplex virus type*, and AdV - *Adenovirus*. ‡Titers are expressed in $\log_{10}$ $TCID_{50}$/25 μL. Each value is the mean titer of three infected wells. *Number of passages. §Absence of viral replication.
Conventional sign used: – Numeric data equal to zero is not due to rounding.

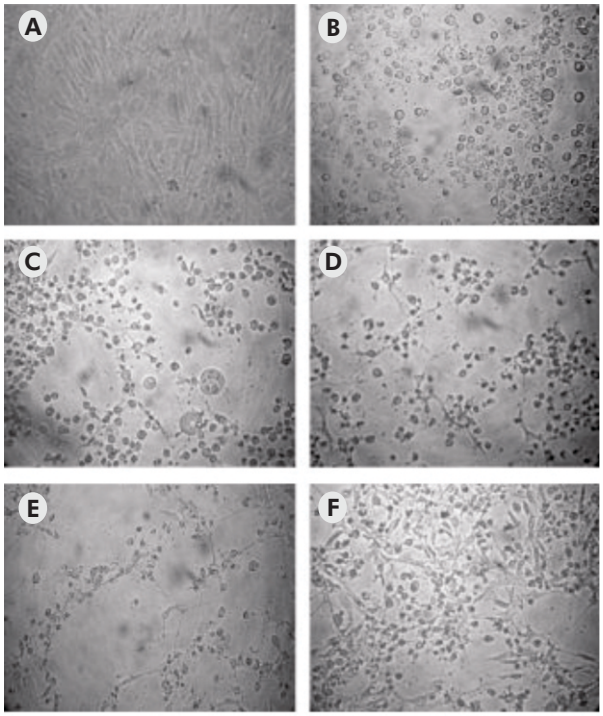

Figure 1 – Cytopathic effect caused by the replication of virus in GRX cells 48 h post-infection. **A**: Control GRX; **B**: HSV-1; **C**: HSV-2; **D**: MAY; **E**: SIN; and **F**: WEE

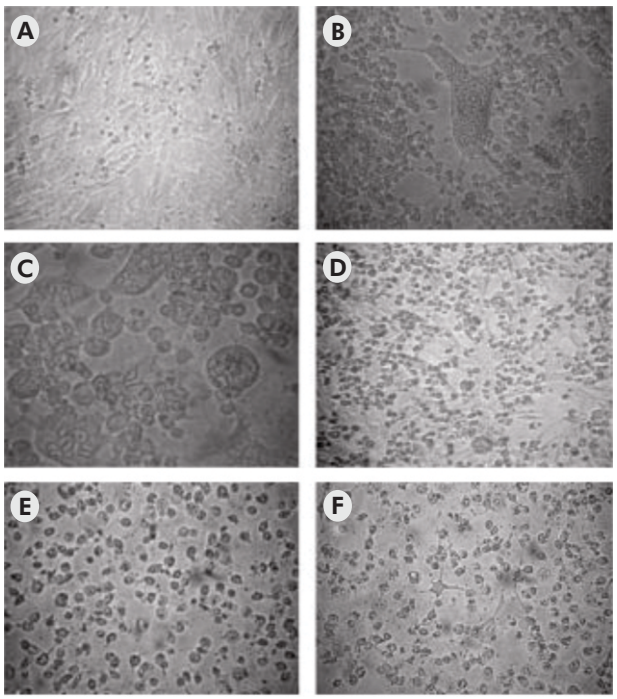

Figure 2 – Cytopathic effect caused by the replication of virus in Vero cells 48 h post-infection. **A**: Control GRX; **B**: HSV-1; **C**: HSV-2; **D**: MAY; **E**: SIN; and **F**: WEE

To demonstrate the efficiency of viral replication, we measured the viral yield in GRX cells compared to that of Vero cells after inoculation with virus at different MOIs. We observed no statistically significant difference between the two cell lines, indicating that viral replication occurs efficiently in both systems (Figure 3). For Vero cells, no significant difference was observed when the viruses were inoculated at different MOIs. For GRX cells, although no significant differences in viral yield were observed for SIN and MAY at different MOIs, we observed some variance in the yield of MAY replication when these viruses were inoculated at a low MOI. Conversely, there was a significant difference ($p = 0.016$) in the viral yield for WEE when different MOIs were used (Figure 3).

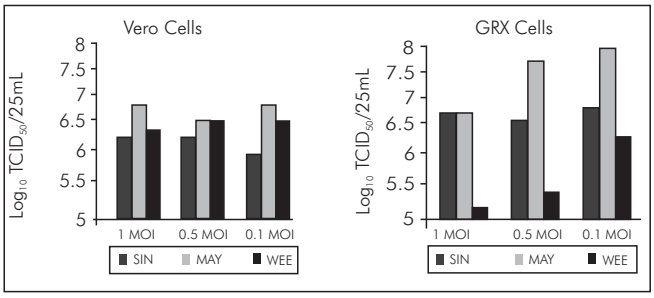

Figure 3 – Measurements of the yield of SIN, MAY, and WEE replication in GRX and Vero cell lines. Each value is the mean titer of three infected wells

Although several cell lines have been used for viral isolation, there are still a significant number of viruses that cannot be propagated in the laboratory. Therefore, the development of a new cell line susceptible to viral replication engenders new alternatives for the cultivation of such viruses. The search for new alternatives has been intensified lately, mainly due to the emergence of novel viruses, such as the human metapneumovirus[7] and the new human coronaviruses (SARS-CoV, HCoV-NL63, HCoV-HKU1)[8,9,10], or to the necessity of viral isolation for biochemical or molecular studies as in the case of norovirus[11], hepatitis B virus[12], and hepatitis C virus[13]. Consequently, studies have been published evaluating the utility of several cell lines for virus isolation[14,15,16].

The GRX cell line was first described in 1985[2]; however, the usefulness of these cells to viral replication is yet to be determined. In this study, we evaluated the ability of the GRX cell line to support the propagation of several viruses and its usefulness as a tool for virology laboratories. Generally, the determination of the susceptibility of a cell line to viral replication can be achieved by observing the characteristic CPE followed by viral titration or by using alternative methodologies, such as a hemagglutination test, immunofluorescence or the detection of the viral genome by molecular methods[14,15,16]. In this study, we used CPE observations and viral titration as the parameters for demonstrating cell susceptibility. All the viruses used in this study produced visible CPE in other cell lines; hence, it would not be advantageous to establish a new cell line for those viruses that require alternative methodologies to demonstrate virus propagation in culture.

The alphaviruses WEE, MAY, and SIN infect a large range of hosts in nature, replicating in mammals, birds, arthropods, and amphibians, and these viruses can be propagated *in vitro* in a great variety of cell lines[17,18,19]. Inoculation of the GRX cells with these viruses produced CPE in 24-48 h. High viral titers were obtained starting with the first passage. The titers increased progressively with consecutive passages, demonstrating the production of infectious virus particles. The GRX cells provided a substrate for viral propagation as efficiently as the Vero cells, which are regularly used for the propagation of alphaviruses.

Different cell lines can be used for the isolation of HSVs[20]. HSV-1 and HSV-2 were successfully propagated in GRX cells at 48 h post-inoculation, showing that although the viral titers obtained were lower than those from Vero cells, GRX cells can be efficiently used as a culture system for these viruses.

Interestingly, in the present study all the viruses that successfully propagated in the GRX cells are enveloped (Alphaviruses) as opposed to non-enveloped viruses, such as adenovirus and rotavirus. One possible explanation for this phenomenon could be the specificity of the viral receptor. Another explanation could be the presence of cellular enzymes required for viral replication. More studies need to be carried out to clarify this issue.

The results showed that the GRX cell line exhibited high susceptibility to different viruses, yielding high titers. Virus-induced CPE was observed between 24 and 48 h, depending on the virus used. The measurement of viral yield demonstrated that the titers progressively increased with the passages, indicating that the GRX cells can efficiently sustain viral replication. The GRX cell line is highly susceptible to HSV-1, HSV-2, MAY, SIN, and WEE, and it can be used as a tool for viral isolation and biochemical studies.

Although there are numerous cell lines widely used in virology to propagate viruses, the establishment of a new cell system always opens new possibilities for exploring the biology and biochemistry of viral infections. The GRX cell line was established in the earliest 1980s, and its biological and biochemical characteristics have been determined. However, its ability to support viral replication has not been clearly demonstrated. The primary goal of this work was to demonstrate the utility of the GRX cell line for viral propagation. For comparison, we worked initially with viruses that already have an efficient cell system. After establishing the GRX cell line's susceptibility to particular viruses, we can continue to study its ability to support the growth of viruses that have not yet been propagated *in vitro*, such as hepatitis C virus and human bocavirus.

In conclusion, (i) we were able to fulfill our primary goal of demonstrating the susceptibility of the GRX cell line to virus infection, (ii) we have shown that this cell line is a valuable virological tool, and (iii) our results open the possibility of using the GRX cell line for viruses not previously propagated *in vitro* by us or by any other laboratory interested in such studies.

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Suscetibilidade de uma linhagem celular murina contínua (GRX) à infecção viral

RESUMO

Este estudo avaliou a capacidade de uma linhagem celular murina (GRX) de realizar a replicação viral. Culturas de células GRX foram infectadas com diferentes vírus DNA e RNA. Foi observado que a linhagem celular GRX é suscetível à replicação dos vírus *Herpes simplex* tipos 1 e 2 (HSV-1 e HSV-2), *Mayaro* (MAY), *Sindbis* (SIN) e vírus da encefalite equina do oeste (WEE) e pode ser utilizada como suporte para estudos sobre replicação viral. A replicação viral induziu o efeito citopático 24 a 48 h pós-infecção. As células GRX produziram titulações de vírus infecciosos entre $10^{2.4}$ $TCID_{50}$ (dose infecciosa de cultura de $tecido_{50}$)/25 µL e $10^{5.4}$ $TCID_{50}$/25 µL na primeira passagem viral. Esses resultados demonstram que as células GRX sustentam, de forma eficiente, a replicação viral e, portanto, podem ser utilizadas como uma ferramenta valiosa para estudos laboratoriais sobre virologia.

Palavras chave: Técnica de cultura de células; Células Estreladas do Fígado (GRX); Replicação Viral.

Susceptibilidad de un linaje celular murino continuo (GRX) a la infección viral

RESUMEN

Este estudio evaluó la capacidad de un linaje celular murino (GRX) de realizar la replicación viral. Culturas de células GRX fueron infectadas con diferentes virus ADN y ARN. Se observó que el linaje celular GRX es susceptible a la replicación de los virus *Herpes simplex* tipos 1 y 2 (HSV-1 y HSV-2), *Mayaro* (MAY), *Sindbis* (SIN) y al virus de la encefalitis equina del oeste (WEE) y puede utilizarse como soporte para estudios sobre replicación viral. La replicación viral indujo el efecto citopático 24 a 48 h pos-infección. Las células GRX produjeron titulaciones de virus infecciosos entre $10^{2.4}$ $TCID_{50}$ (dosis infecciosa de cultura de $tejido_{50}$)/25µL y $10^{5.4}$ $TCID_{50}$/25 µL en el primer pasaje viral. Estos resultados demuestran que las células GRX sostienen, de forma eficiente, la replicación viral y, por lo tanto, pueden ser utilizadas como una valiosa herramienta para estudios de laboratorio sobre virología.

Palabras clave: Técnicas de Cultivo de Célula; Células Estrelladas Hepáticas (GRX); Replicación Viral.

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