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Original Article

Purification of Human Reovirus in Monolayer of L-929 Cells

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Abstract

Background and Aims: The ability of the reovirus to destroy various cancer cells in vitro and, the success of the virus in conducting clinical trial phases of cancer therapy has attracted the attention of researchers toward the virus. One of the main needs for the investigation of the viral effects and the virus-host cell interaction is preparation of the purified virus. Most of the protocols have been based on the use of suspended cell culture equipment that normally does not exist in the research laboratories. We optimized a virus purification method that was based on using cell culture flask and adherent cells.

Materials and Methods: L-929 cells were used for reovirus propagation. After sufficient CPE, the cells were collected and pelleted. Using Vertrel-XF treatment and ultracentrifugation on the cesium chloride (CsCl) gradient, purified reovirus was obtained. It was subsequently concentrated by filtration using a 100kDa Amicon unit.

Finally, infectivity and the number of purified human reoviruses were evaluated by plaque assay. The band of purified human reovirus was aspirated form the ultracentrifugation tube and then was dialysed and concentrated by filtration in Amicon unit. The titer of purified human reovirus was determined to be 3×1012 PFUs/ml.

Results: In present study, we established a protocol for the purification of human reovirus without need for equipment of suspension cell culture.

Conclusions: Although, the time-consuming dialysis procedure was removed from the end of the work and replaced with a rapid and simple filtration method, the high titer of purified human reovirus was acquired.

Keywords: oncolytic virus, reovirus, virus purification, CsCl gradient.

Introduction

Reovirus is one of the six genera of the family Reoviridae which can infect a wide range of creatures, such as mammalians, birds, plants, and insects (1, 2). In 1953, Stanley et al, isolated the virus for the first time from the stool specimen of a native child inhabitant at Yarra Bay Aboriginal settlement in Sydney of Australia. Various experiments on newborn mice showed that reovirus caused oily hair effects, encephalomyelitis and other viral diseases. Hence, it was incipiently named hepato-encephalomyelitis virus. In 1959, Albert B Sabine used the term reovirus to describe this group of cytopathogenic viruses (3, 4). Roe's term is abbreviated Respiratory Enteric Orphan. These viruses are often isolated from the respiratory and enteric tract of humans without clinical symptoms or with mild clinical symptoms (5).

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Genome in these viruses includs 10 segments of double-stranded RNA-surrounded by a spherical capsid with icosahedral symmetry (6).

The dsRNAs are divided into 3 class based on their movement rate on the gel electrophoresis: large segments with size of about 3854-3916 bp (L1, L2, L3), medium segments with size of about 2206-2304 bp (M1, M2, M3) and small segments with size of about 1196-1416 bp (S1, S2, S3, S4) (7, 8).

There are four serotypes of reovirus based on inhibition of hemagglutination and neutralization: type 1 Lang, type 2 Jones, type 3 (Abney and Dearing) and type 4 Ndelle Which has recently been isolated from a mouse in Cameroon (5, 9).

These serotypes differ significantly in response to a variety of physical and chemical inactivity factors, host cell tropism, mechanisms of cell death, CNS disease and method of diffusion (8). Reoviruses are found everywhere in the environment; such as sewage and surface water. For this reason, 70-100% of adult persons aged between 20 to 30 years are exposed to this virus during their lifetime. So, they have an antibody against this virus which makes them safe from reovirus infections (10, 11).

Reoviruses are one of the oncolytic viruses that can lyse cancer cells through their oncolytic process (12). Activated ras pathway helps reoviruses to be able to replicate in and destroy various cancer cells. Activation and overexpression of Ras can be due to a direct mutation in ras proto-oncogene or mutation in the upstream and downstream elements of the ras gene. These mutations play an important role in growth, angiogenesis, and metastasis of the tumors (13, 14). In these cells, the early transcript of the RNA virus is translated by phosphorylation and inactivation of double-stranded RNA dependent protein kinase (PKR). After replication of the virus, the lytic cycle occurs and ultimately precedes the death of the cell. While in normal cells, activation of PKR prevents translation of the early transcript of the RNA virus, the lytic cycle stops and the cell survives (15-18).

Considering two properties of reoviruses (non-pathogenic and oncolytic), these viruses can be used as a therapeutic agent against various cancers. According to this, replication and purification of these viruses have particular importance in the different field of molecular researches. Herein, we optimized a simple, inexpensive and least experimental facility for purification of the reovirus.

Methods

Propagation of human reovirus. Reovirus T3D was kindly provided by Dr. Ataei-Pirkooh (Iran University of medical sciences). Fifty 75-cm3 cell culture flasks were prepared and L-929 cells. L-929 cells were seeded in all flasks followed by adding supplemented culture medium containing Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin. Cell culture flasks were incubated at 37ºC in a humidified 5% CO2 incubator until cell confluence of ~80%. Culture medium was then poured off followed by two washes with phosphate buffered saline (PBS). Subsequently, L-929 cells were infected with human reovirus at an MOI of 10 PFUs/cell and incubated 1 hour at room temperature for virus adsorption (flask was gently rocked every 15 minutes to prevent the cells from drying out). Then the culture medium without FBS was added to the cells and were incubated at 37°C until ~80% cytopathic effect (CPE).

Purification of human reovirus. After ~80% CPE, the cells were gently collected using 29-cm cell scraper (SPL Life Sciences, South Korea). Afterward, collected cell-virus mixture were centrifuged at 5,000 g for 30 minutes at 4°C.

The supernatant was poured off and pelleted cells were collected in two conical tubes. The pellets were resuspended in

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stabilization buffer containing 150 mM NaCl, 15 mM MgCl2 and 10 mM Tris-HCl pH 7.4 and kept in -70°C until the next step. After thawing, pellets were sonicated using a small probe followed by incubation with 10% sodium desoxycholate at room temperature for 20 minutes. Then 2/5th of the total volume of Vertrel XF (Sigma-Aldrich Chemie GmbH, Germany) was added to each tube followed by sonication. Afterward, the suspension was centrifuged at 9000 g for 10 minutes and the top aqueous phase transferred into a fresh tube. Nine -tenth the total volume of Vertrel XF was added followed by mixing and centrifugation the same as the previous step. The aqueous phases were removed onto a freshly prepared CsCl gradient ranging 1.2-1.44 g/ml in an ultracentrifuge tube. Ultracentrifugation was done at 30,000 g for 3 hours followed by the collection of the lower virus band. Then the collected virus band was concentrated using the 100KDa Amicon Filter unit (Millipore, USA). The purified virus was then collected and kept in stabilization buffer plus 15% glycerol at -70°C.

Titration of the purified human reovirus. To quantify and evaluate the infectivity of purified human reovirus, the plaque assay was performed. Briefly, L-929 cells were grown in 6-well cell culture plate and infected by serial dilution of purified reovirus as described in the previous section. After 90 min incubation at room temperature, overlay medium containing DMEM, antibiotic and 0.3% agarose was added into the wells. Seventy-two hours later, the cells were fixed and stained with crystal violet. Plaque assay was done in duplicate.

Results

Propagation of human reovirus. L-929 cells are the best to propagation human reovirus. Human reovirus produced apparent CPE in ~80% of the cells at about 72 hours. Reovirus CPEs including rounding, swallowing and sloughing have been shown in figure 1.

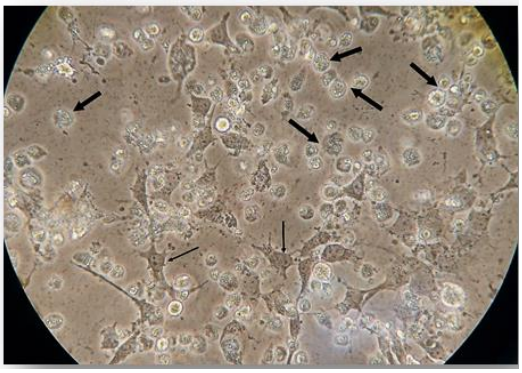

Fig. 1. L-929 cells infected with human reovirus. Thick arrows refer to the cells with CPE and the thin arrows refer to the cells without virus CPE.

Purification of the human reovirus. After ultracentrifugation of the cells lysate, a separated grey band including whole virions was visible in the CsCl gradient solution (Figure 2). The virus band was collected by top aspiration into a 100KDa Amicon Filter unit. After three washes with stabilization buffer, glycerol (final concentration of 15%) was added to the purified human reovirus and then aliquoted and kept at -70°C.

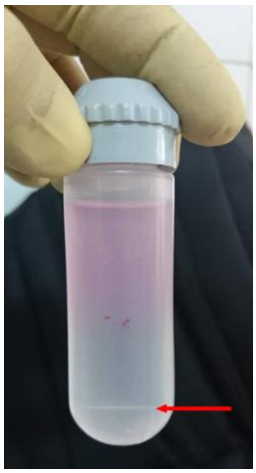

Fig. 2. Ultracentrifugation tube and the band of whole virions of the human reovirus.

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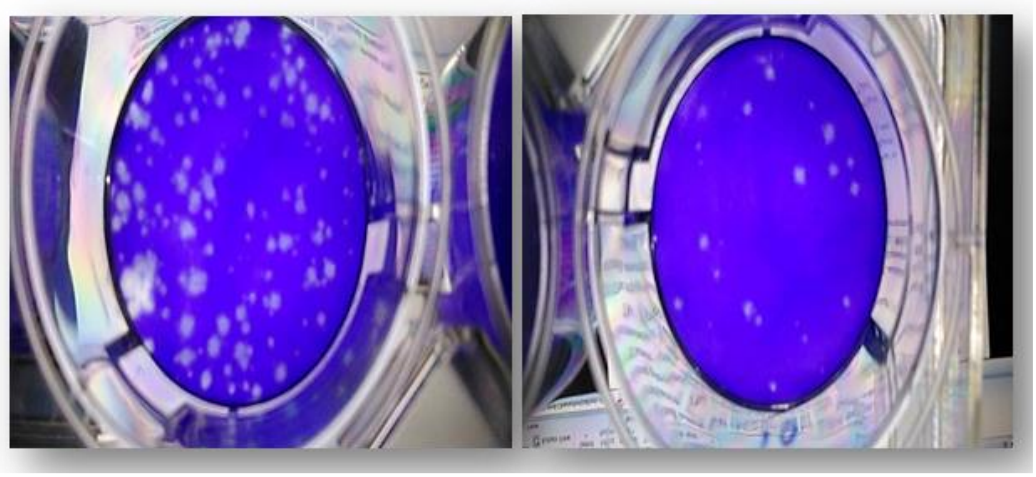

Fig. 3. Plaque assay of the purified human reovirus. The left picture is high viral concentration and the right is low and countable.

Titration of the purified human reovirus. Each plaque is a representative of a whole virion. The viral plaque was visible as a clear (Figure 3) zone, these clear zones were counted, and the final number of virions were calculated. We were able to obtain 3×109 PFUs/ml of purified human reovirus, however, this number could be increased by decreasing the volume of the final wash.

Discussion

Purification of viruses from virus-infected cells is one of the most important steps in many structural and biochemical studies (19).

Several methods are usually used to the preparation of viruses for experiments: Density gradient ultracentrifugation, pelleting viruses by centrifugation, ultrafiltration, and chromatography. Density radient ultracentrifugation is the most common method for separating a number of viruses that includes adenovirus, arterivirus, bacteriophage (phage), calicivirus, herpesvirus, papillomavirus, polyomavirus, and reovirus.

Often gradients of CsCl, sucrose, or iodixanol is used for the isolation of viruses from host cells (20).

Hitherto, many studies have shown that the oncolytic properties of reoviruses in various cancers (12, 21-37). For this reason, purification of reoviruses is of particular importance. In typical methods of reovirus purification, L-929 cells were cultured as suspension (38).

Accordingly, this method was depended on the specific equipment such as specific incubators and spinner flasks that is not found in any laboratory. However, in the present study, L-929 cells were cultured monolayered form and common cell culture flasks were utilized for purification of reoviruses. Also, this method can be done in a short time, without doing dialysis step and using Freon.

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