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Growth of a murine coronavirus in a microcarrier cell culture system

Pierre J. Talbot[1], Jacques Lapierre[2], Claude Daniel[1], Robert Dugré[2] and Pierre Trépanier[2]

[1]*Centre de recherche en virologie and* [2]*Division des vaccins viraux, Institut Armand-Frappier, Université du Québec, Ville de Laval, Québec, Canada*

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Summary

The growth of the murine coronavirus MHV-A59 on murine DBT cells adapted to dextran-made Cytodex 1 microcarriers was studied in comparison with cells grown on plastic dishes. With a microcarrier concentration of 5 g/l in spinner flasks, a density of 3×10^6 cells/ml was reached in 7 days. Under these conditions, cells supported virus growth to the same extent as when they were grown on the plastic substratum. This was shown by a similar development of virus-induced syncytia, the release of an equivalent number of infectious progeny virions per cell, similar recoveries observed after concentration and purification and an identical appearance of the purified virus under the electron microscope. On the other hand, the technical convenience of microcarriers and the ease of scale-up emphasize their potential for the growth of coronaviruses.

Coronavirus; Microcarrier; Cell culture

Introduction

Coronaviruses are the etiological agents of various acute and chronic diseases of mammals and fowl (Wege et al., 1982; Siddell et al., 1983). They are enveloped viruses of 80 to 120 nm in diameter and contain single-stranded RNA of positive polarity. In mice and rats, several strains of murine hepatitis viruses (MHV) are

Correspondence to: Pierre J. Talbot, Institut Armand-Frappier, Université du Québec, 531, boul. des Prairies, Ville de Laval, Québec, H7V 1B7 Canada.

responsible for neurological, respiratory and gastrointestinal disorders. We are studying the neurotropic A59 and JHM strains of MHV, as animal models of virus-induced neurologic disease, with possible relevance to human disorders. Molecular determinants of pathogenesis were located on the viral spike glycoprotein E2 (Talbot et al., 1988a and b). To characterize further this protein, the protein was purified from MHV-A59 virions by affinity chromatography from concentrated virus produced on DBT cell monolayers grown in 150 cm^2 dishes (manuscript in preparation). However, hundreds of such dishes are required to obtain sufficient amounts of purified E2, which is both expensive and technically tedious. The possible use for cell culture of small globular spheres or microcarriers held in suspension by gentle stirring would represent an alternative approach to the proliferation of dishes, flasks or roller bottles. The impressively large surface area generally available for cell growth with microcarriers permits the production of large number of cells in a relatively small volume. Microcarriers with a dextran matrix were initially used by Van Wezel (1967, 1973) and were later modified by Levine et al. (1977) to eliminate their reported toxicity. Subsequently, other materials have also been used as microcarriers: glass (Varani et al., 1983), polystyrene plastic (Johansson and Nielsen, 1980), collagen, porous silica and polyacrylamide (Gebb et al., 1982) -made microcarriers were all reported to support the growth of anchorage-dependent cells.

Dextran-made microcarriers were used to evaluate the feasibility of adapting the murine DBT cell line for growth and to produce virus. The pilot study showed that DBT cells grew efficiently on these microcarriers and that viral titers obtained were equivalent to those obtained with cells grown in plastic dishes.

Materials and Methods

Virus and cells

The A59 strain of MHV and the DBT murine cell line were obtained and grown and viral infectivity quantitated as described previously (Daniel and Talbot, 1987).

Growth of DBT cells on microcarriers and viral infection

Cytodex I microcarriers (Pharmacia, Montréal, Québec, Canada) were used and prepared according to the manufacturer's instructions. Briefly, the dry microcarriers were swollen and hydrated overnight in Ca^{2+} and Mg^{2+}-free phosphate buffered saline at room temperature. The microcarriers were then washed twice in the same buffer and subsequently sterilized by autoclaving. Before use, the beads were rinsed briefly in warm cell culture medium.

All experiments were performed in 250 ml siliconized spinner flasks (Bellco Glass, Inc., Vineland, New-Jersey, U.S.A.). Cell and microcarrier concentrations used per flask were 85×10^6 cells and 1.25 g (5 g/l), respectively. The medium used was L-15 supplemented with 5% Cv/v fetal calf serum and 100 μg/ml of kan-

amycin sulphate. Microcarrier cultures were stirred at 50 rpm using a microcarrier magnetic stirrer (Bellco Glass Inc.) and medium was changed daily. For infection, virus was added at a multiplicity of infection (MOI) of 0.01 when cell confluency was observed on the microcarriers. After complete cell destruction, viral supernatants were collected and processed as described below. Cells were enumerated by counting nuclei as described by Levine et al. (1977).

For comparison, cells grown to confluence on plastic 150 cm^2 culture flasks (Corning Glass Works, Corning, NY, USA) were similarly infected, although the culture medium was as described previously (Daniel and Talbot, 1987).

Virus concentration and purification

The growth medium of infected cells on microcarriers or on plastic flasks was collected by decantation or aspiration, respectively. The viral suspension was clarified by centrifugation (10000 × *g*) at 4°C for 20 min and brought to 0.5 M NaCl and 10% (w/v) polyethylene glycol (PEG) 8000 (Sigma, St-Louis, MO, USA). After overnight incubation at 4°C with gentle stirring, the virus concentrate was collected by centrifugation at 10000 × *g* for 30 min and resuspended in 3.5 ml TMEN buffer (0.1 M Tris acid maleate, pH 6.2, 1 mM ethylene diamine tetracetic acid and 0.1 M NaCl). This PEG-concentrated virus was layered on a discontinuous 10 and 50% (w/v) Nycodenz® (Nyegaard, Oslo, Norway) gradient in TMEN buffer and ultracentrifuged at 83000 × *g* for 3.5 h in a SW 28 rotor at 4°C. The gradient was then fractionated from the bottom and virus-containing fractions identified by electron microscopy, pooled and diluted 2-fold with TMEN. Further purification was achieved on a continuous 10–50% (w/v) gradient ultracentrifugation for 16 h. Virus-containing fractions were pooled and dialyzed against TMEN buffer.

Electron microscopy

After direct sedimentation of the samples on grids and negative staining (Alain et al., 1987), the samples were examined with a Philips EM 300 electron microscope.

Results

A typical growth curve of DBT cells on the dextran made Cytodex I microcarriers is shown in Fig. 1. Cells multiplied rapidly for the first 6 days before reaching a plateau. A cell concentration of 3×10^6 cells/ml was generally obtained in 7 days. Cell infection was always performed on confluent beads which were obtained 6 or 7 days after seeding. The morphological aspect of the cells on microcarriers is shown in Fig. 2. Confluent beads showed elongated cells covering all of the available surface on the microcarriers (Fig. 2A, B). Infected cells showed complete cytopathic effect (syncytia) within 18 hrs p.i. and detached completely from the microcarriers (Fig. 2C, D).

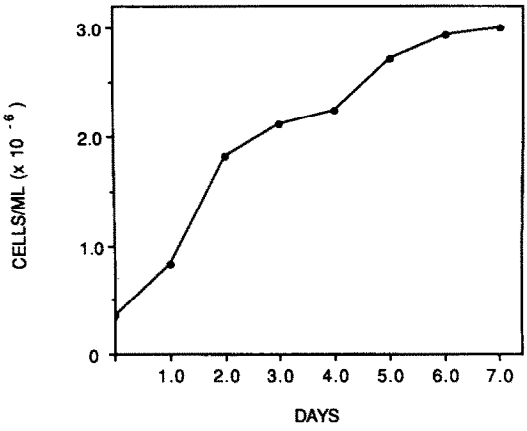

Fig. 1. Growth curve of DBT cells on Cytodex I microcarriers in a 250 ml spinner flask. Enumeration of cells by counting nuclei.

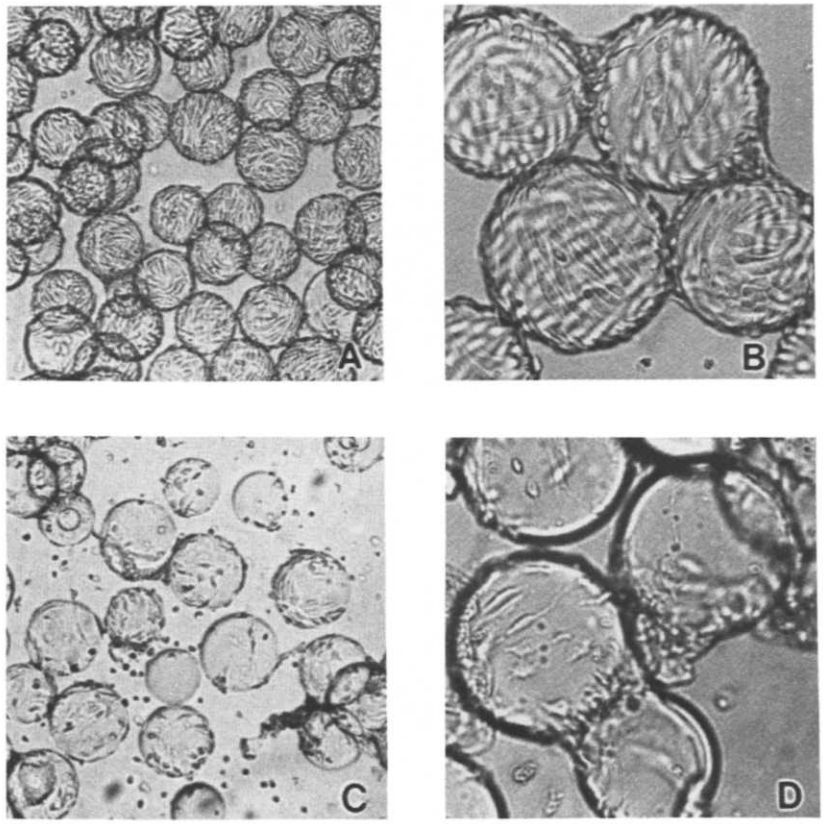

Fig. 2. Photographs showing uninfected and confluent DBT cells on microcarriers at day 7 (A, B). Infected cells (C, D) shown at 18 h p.i. and detaching from the beads. Magnification: A, C: 50×; B, D: 125×.

TABLE 1

Comparison of microcarriers to culture dishes for the growth of MHV-A59 on DBT cells

Cell substratum	Medium volume (ml)	Number of cells		Infectivity[a] (PFU/ml)		
		per ml	total	per ml	total	per cell
Microcarriers	250 ± 10	$3.0 \pm 0.3 \times 10^6$	$7.5 \pm 1.1 \times 10^8$	$1.3 \pm 0.4 \times 10^7$	$3.2 \pm 1.2 \times 10^9$	4.3 ± 2.2
Culture dishes[b]	165 ± 11	–[c]	$5.5 \pm 0.3 \times 10^8$	$2.0 \pm 0.5 \times 10^7$	$3.3 \pm 0.8 \times 10^9$	6.0 ± 1.8

[a]Infectious virus released 18 h post-infection at MOI = 0.01.
[b]Eleven 150 cm^2 plastic flasks.
[c]No cells in medium; all are attached on substratum.

TABLE 2

Comparison of microcarriers to culture dishes for the concentration and purification of MHV-A59 released from DBT cells

Cell growth	Sample	Volume (ml)	Infectivity (log_{10} PFU)		Recovery[a] (%)
			per ml	total	
Microcarriers	Medium	250	7.1	9.5	100
	PEG concentrate	3.5	8.3	8.8	20
	Purified virus	1.4	7.0	7.2	0.5
Culture dishes[b]	Medium	165	7.3	9.5	100
	PEG concentrate	3.5	8.2	8.8	20
	Purified virus	4.7	6.1	6.8	0.2

[a]Recovery of infectivity from medium.
[b]Eleven 150 cm^2 plastic flasks.

Infectious virus released from DBT cells grown on microcarriers was quantitated by plaque assay and the titer compared to what was obtained from cells grown on culture dishes. The results are shown in Table 1. There was no statistically significant difference in the release of infectious virus per cell. Moreover, a single small 250 ml flask of infected cells on microcarriers released as many infectious virions as eleven 150 cm^2 dishes. When virus was concentrated and purified from the growth medium of infected DBT cells on microcarriers, similar recoveries of infectivity were observed as with virus obtained from cells grown on culture dishes (Table 2). Moreover, the appearance of purified coronavirus in the electron microscope was indistinguishable (Fig. 3).

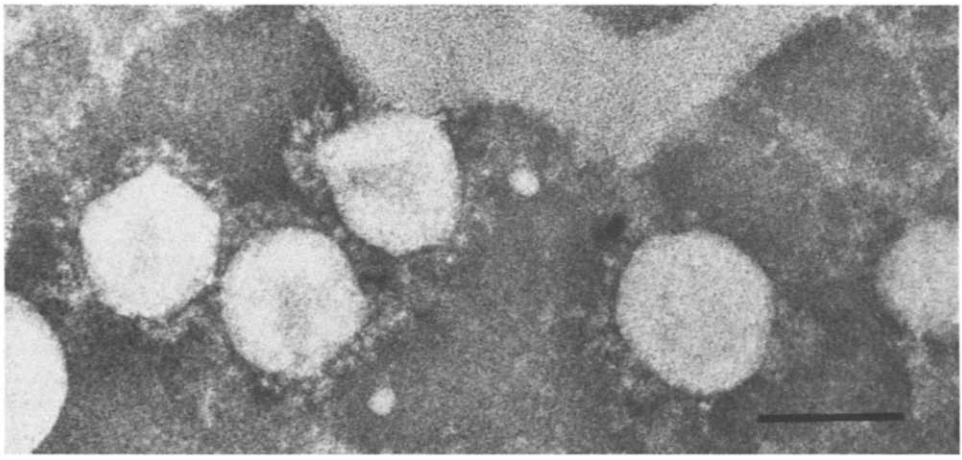

Fig. 3. Typical MHV-A59 virions after purification from the growth medium of infected DBT cells grown on microcarriers. An aliquot was examined by electron microscopy under negative staining, as described in Materials and Methods. Bar represents 100 nm.

Discussion

This pilot study was aimed at evaluating the possibility of growing DBT cells in a microcarrier culture for use in the propagation of a murine coronavirus. The results show that DBT cells could be successfully grown on Cytodex I microcarriers. Using a microcarrier concentration of 5 g/l, a density of 3×10^6 cells per ml was obtained after 7 days. This is similar to various other cell types that were used on microcarriers, as reported before (Anonymous, 1981). Cell morphology was identical on microcarriers as on plastic dishes. This suggested that conditions were optimal for testing viral replication. When MHV was introduced into the microcarrier culture at an MOI of 0.01, virus replicated rapidly and cell destruction was complete within 18 h. However, virus-induced syncytia detached from the microcarriers whereas the latter remained attached on plastic flasks. It is likely that the stirring of microcarriers led to the release of syncytia from their surface. Nevertheless, a similar viral titer of more than 10^7 PFU/ml was obtained in both culture conditions. The virus was similarly amenable to concentration and purification and showed an identical morphology in the electron microscope. Thus, the growth of DBT cells on microcarriers did not affect the replication of this murine coronavirus compared to plastic dishes, as monitored by cytopathic effect, output of infectious virus per cell, its behavior upon concentration and purification and its morphological appearance.

The advantages of using a microcarrier culture over plastic dishes or flasks are numerous. In the former, labware is reduced and it is much less time consuming than more conventional culture techniques. Laboratory manipulations are also reduced, thus diminishing risks of contamination. Moreover, under the present experimental conditions, comparable virus yields were obtained. The optimization of media supplements and gas exchange found in a perfused microcarrier culture should allow an increase in microcarrier concentration and ultimately permit a higher density of cells to produce even more virus particles.

The growth of coronavirus on microcarrier cultures provides a very efficient approach for large scale production of such viruses, which should greatly facilitate detailed biochemical studies of these important pathogens. We are currently applying this technology to the molecular analysis of the spike glycoprotein E2 of murine coronaviruses, an important model for virus-induced neurological diseases. Microcarrier cultures should also prove invaluable for the study of poorly replicating viruses, as well as for the large scale production of viral vaccines, including the expression of molecularly cloned viral genes.

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