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Virus Production with a Newly Developed Microcarrier System

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Primary cell cultures as well as established lines have been grown on a recently developed microcarrier configuration that overcomes the problem of toxicity attendant on earlier developments in this technology. Virus yields from these cells propagated on the new microcarriers have been measured. Microcarrier-grown cells, when compared to roller-bottle-grown cells, gave virus yields on a per-cell basis that varied from slightly greater with the Sindbis virus-Chinese hamster ovary cells and polio-WI-38 combinations to approximately one-third with Moloney murine leukemia virus-Cl-1 mouse cells and vesicular stomatitis virus-chicken embryo fibroblasts. Yields ranged from 8.0×10^7 to 3.6×10^8 cells per 100-ml microcarrier culture and from 3.7×10^7 to 4.1×10^8 cells per roller-bottle culture. Secondary chicken embryo fibroblast yields were approximately four times as great in microcarrier cultures as in standard roller-bottle cultures, per unit volume of medium consumed. In spite of the reduced virus yields per cell seen in some instances, the greater cellular productivity of microcarrier cultures appears to hold great promise for large-scale virus production. Optimizing microcarrier conditions for specific cell-virus systems should result in improved yields.

Until recently, almost all mass production of anchorage-dependent cells that could be used for virus production was accomplished with vessels such as prescription bottles or roller bottles (3, 4). This method is laborious and expensive and in many cases caused biohazard problems difficult to deal with. Some progress was achieved with the development of the multisurface stacked-plate propagator (14) and spirals of plastic fiber (2). The work of van Wezel (11-13), who used anion-exchange resin as a microcarrier to grow surface-dependent cells in suspension, was encouraging, but subsequent developmental work (D. W. Levine, D. I. C. Wang, and W. G. Thilly, *in Proceedings of the First Annual Cell Culture Congress,* Birmingham, Ala., 1975, in press; 1, 9, 10, 13) failed to produce a generally satisfactory microcarrier configuration.

More recent developmental work with microcarriers in our laboratories (5) has apparently overcome the problems of toxicity associated with microcarriers; this provides not only a practical means for mass propagation of cells, but also the potential for mass producing viruses and a wide variety of cell products. This communication reports the results of our first small-scale studies with this newly developed microcarrier system for the production of viruses.

MATERIALS AND METHODS

Cells and viruses. Secondary chicken embryo fibroblasts (CEF) were produced from 11-day-old SPF-COFAL eggs (SPAFAS, Inc., Norwich, Conn.). Chinese hamster ovary (CHO) cells were obtained from Stuart Kornfeld, University of Washington, St. Louis, Mo. Murine leukemia virus (MuLV)-infected NIH-3T3 cells (Cl-1) were obtained from David Baltimore, Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Mass. African green monkey kidney cells (AGMK) were supplied by Microbiological Associates, Bethesda, Md., and WI-38 cells were obtained from the American Type Culture Collection, Rockville, Md. The established cell line of AGMK (CV-1) was obtained from Melvin DePamphilis, Harvard Medical School, Boston, Mass., and FS-4 cells were provided by Jan Vilček, New York University School of Medicine, New York, N.Y. Sindbis virus (HR strain) was obtained from Steve Harrison, Harvard Medical School, Boston, Mass. Vesicular stomatitis virus (VSV; Indiana strain) was obtained from David Baltimore, and poliovirus (Leon strain, type 3) was supplied by the American Type Culture Collection.

Culture vessels. Plastic roller bottles (Corning Glass Works, Corning, N.Y.) and 250-ml glass spinner bottles equipped with a 4.5-cm, magnetically driven, Teflon-coated stir bar (Wilbur Scientific, Inc., Boston, Mass.) were used for cell production.

Media. Sera were obtained from K. C. Biologicals, Lenexa, Kans. (calf serum), Microbiological Associates (chick serum), and Flow Laboratories, Rockville, Md. (fetal calf serum). Dulbecco modified Eagle medium was supplied by Flow Labs, and trypsin (1:250), Noble agar, and tryptose phosphate broth were supplied by Difco Laboratories, Detroit, Mich. Dulbecco modified Eagle medium was supplemented with penicillin (100

U/ml) and streptomycin (100 μg/ml). Growth medium consisted of Dulbecco modified Eagle medium supplemented with 1% calf serum, 2% tryptose phosphate broth, and 1% chick serum for CEF, 10% fetal calf serum for CHO, AGMK, WI-38, and FS-4, and 10% calf serum for Cl-1 cells. Medium for virus production consisted of Dulbecco modified Eagle medium supplemented with 1% calf serum and 2% tryptose phosphate broth (CEF), 2% fetal calf serum (CHO and WI-38), and 10% calf serum (Cl-1). Agar overlays for plaque assays consisted of minimum essential medium (Microbiological Associates) with 1.5% agar, supplemented with 1% calf serum and 2% tryptose phosphate broth (CEF) and 2% fetal calf serum (CV-1 cells).

Microcarrier preparation. The initiation of microcarrier cultures has been described elsewhere (5) but consisted essentially of the following. Microcarriers were suspended in phosphate-buffered saline at a concentration of 10 mg/ml and were sterilized in glass bottles by autoclaving. Microcarriers were then dispensed into spinner flasks containing growth medium to give a final concentration of 5 mg/ml. The procedure for microcarrier preparation has also been described previously (5).

Cell production. Cell stocks grown in roller bottles were washed with phosphate-buffered saline, dissociated with 0.1% trypsin, and counted in a hemacytometer before seeding. Cells were seeded simultaneously in spinner flasks containing microcarriers and in roller bottles, and allowed to grow for approximately 1 week. Microcarrier cultures were seeded at approximately 2.5×10^5 cells per ml (1.4×10^4 cells per cm^2) in a 100-ml volume. This concentration appeared to be satisfactory for all cell types tested. Roller bottles were seeded at 1×10^5 to 2×10^5/ml in a 100-ml volume (2×10^4 to 4×10^4 cells per cm^2). Cells were allowed to achieve confluency and then were allowed an additional 2 to 3 days to permit cells in each type of vessel to become as dense as possible. All cultures were grown at 37°C. In both systems, the pH was initially adjusted to approximately 7.4, and a gradual drop was observed during the course of cell growth.

Virus infection and production. All cell types, with the exception of Cl-1 (a spontaneous producer of MuLV), were infected after cells had reached maximum density. A multiplicity of infection of 0.05 was used for Sindbis virus and 0.1 for poliovirus and VSV. An adsorption period of 1 h was allowed for each virus (5 ml of virus suspension per roller bottle and 25 ml per microcarrier culture), after which the volume of medium in each vessel was brought up to 50 ml. Cultures were incubated at 37°C. Roller bottles were rotated at a rate of 0.5 rpm. Microcarrier cultures were adjusted to a stirring rate of approximately 80 rpm. The pH in both systems was adjusted to approximately 7.4. Virus fluids were collected after 24 h, clarified by centrifugation at 2,000 rpm at 4°C, and titrated by plaque assay.

Virus plaque assays. Sindbis virus and VSV were titrated in secondary CEF, and poliovirus was titrated in CV-1 cells. Serial 10-fold dilutions were made with each virus sample, and 0.2 ml was inoculated in duplicate onto monolayers of cells grown in petri dishes (60 by 15 mm; Falcon Plastics, Oxnard, Calif.). A 1-h adsorption period was allowed at 37°C in a 10% CO_2 atmosphere. Dishes were frequently rocked to prevent drying out and to allow an even distribution of virus particles. Each dish received 4 ml of agar overlay, and dishes were incubated at 37°C. Plaques were read at 48 to 72 h postinfection with a 1:2,500 dilution of neutral red (Fisher Scientific Co., Medford, Mass.). The procedure for assaying Moloney MuLV has been described elsewhere (6).

Determination of medium requirements for cells grown in roller bottles and on microcarriers. To compare cell yields per unit volume of medium consumed, roller bottles and microcarrier flasks were seeded with CEF, and growth curves were done over an 8-day period. Roller bottles were divided into three groups, which received 25, 50, and 100 ml of growth medium per bottle. All bottles received 1.6×10^7 cells (1:4 split). Each day, duplicate samples of each group were dispersed with trypsin, and cell counts were performed. Microcarrier cultures (100 ml) were set up at 2.5×10^7 cells per flask, and daily cell counts were made. These 100-ml microcarrier cultures and the 100-ml roller-bottle cultures were given a medium change on day 4 by removing 50 ml of spent medium and replacing it with 50 ml of fresh medium. The total number of new cells produced per milliliter of medium used was calculated for each system after a total of 6 days of growth.

Cell counts. Cells in microcarrier cultures were enumerated by counting nuclei by a modification of the method of Sanford et al. (7) as described by van Wezel (13). Roller-bottle cell counts were made by dispersion with 0.1% trypsin, followed by counting with a hemacytometer.

Quality control. All cultures were thoroughly screened for the presence of mycoplasma by using both the culture method (isolation on artificial media) and the uridine-uracil assay described by Schneider et al. (8). Results were negative on all cells used in this study.

RESULTS

Cell growth. Typical microcarrier growth curves obtained with a variety of cell types are shown in Fig. 1. Cell densities ranged from approximately 1×10^6/ml (1×10^8 per 100-ml culture) with AGMK cells to approximately 3×10^6 cells per ml (3×10^8 per 100-ml culture) with CHO and Cl-1 cells. Lag time before logarithmic growth varied from 48 h with FS-4 and AGMK cells to 24 h with the other cell types. The rate of growth was also markedly varied, ranging from an 18-h doubling time for CHO and Cl-1 cells to approximately 48 h for AGMK cells. Cell attachment occurred within a few hours, and cells were uniformly distributed over the microcarrier surface. In all cases, complete monolayers were formed on at least 95% of the microcarriers, but CEF, CHO, and Cl-1 cells became more densely packed than did the AGMK or FS-4 cells, resulting in greater yields.

Cell and virus yields. Table 1 gives comparative cell and virus yields from 100-ml microcarrier cultures and roller bottles. The values ranged from 3.7×10^7 to 4.1×10^8 cells per roller

bottle and from 8.0×10^7 to 3.6×10^8 cells per 100-ml microcarrier culture. To facilitate direct comparison of the two culture systems (roller bottle and microcarrier culture), the number of roller-bottle equivalents (RBE) per liter of microcarrier culture was calculated both on a cell yield and a virus yield basis. These figures appear in the two right-hand columns of Table 1. Expressed in this manner, the cell growth values ranged from 9 to 48 RBE/liter. The lowest value (9 RBE/liter of microcarrier culture) was observed with Cl-1 cells, which appeared to form multilayers in roller bottles to a greater degree than on microcarriers. The highest values occurred with diploid fibroblasts (CEF, FS-4) and epithelial cells (AGMK). These cells tended to be relatively contact inhibited and had less tendency to form multilayers on either roller bottles or microcarriers than established lines such as CHO and Cl-1.

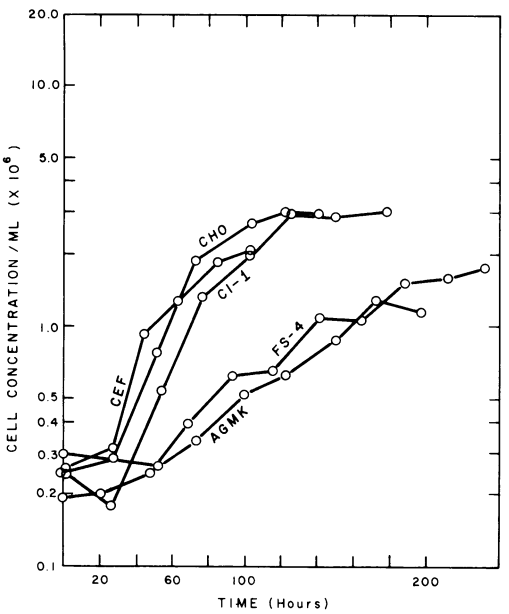

FIG. 1. *Growth of various cell lines on microcarriers. Cells were seeded at approximately 2 × 10⁵ to 3 × 10⁵/ml in 100-ml volumes, with a microcarrier concentration of 5 mg/ml. Medium was replenished after 3 to 4 days by allowing the microcarriers to settle, removing 50 ml of spent medium, and replacing it with 50 ml of fresh medium.*

Virus yields, expressed as plaque-forming units per 10^6 cells, differed considerably between roller bottles and microcarrier cultures. Yields were slightly higher with microcarrier cultures using the Sindbis-CHO and polio-WI-38 systems and considerably lower with the MuLV-Cl-1, VSV-CEF, and Sindbis-CEF systems. Values ranged from 3 RBE/liter (MuLv-Cl-1) to 35 RBE/liter (Sindbis-CHO).

Growth medium requirements. Cells growing in roller bottles and microcarrier cultures were compared with respect to cell yields per unit volume of medium consumed (Table 2).

TABLE 2. *Comparison of culture medium requirements for CEF grown in roller bottles and on microcarriers*

Vessel	Vol of medium	Cells seeded ($\times 10^7$)	Maximum yield ($\times 10^7$)	New cells produced per ml of medium ($\times 10^5$)
Roller bottle (490 cm²)	25	1.6	2.1	2.8
	50	1.6	2.9	2.4
	100	1.6	3.9	2.9
	150[a]	1.6	5.2	2.4
Microcarrier culture (3,000 cm²)	100	2.5	12.0	9.5
	150[a]	2.5	21.0	12.3

[a] Cultures were initially seeded in a 100-ml volume. After 4 days, 50% of the spent medium was replaced with fresh medium.

TABLE 1. *Cell and virus yields from microcarrier and roller-bottle cultures*

Virus-cell system	Cell yield from 100-ml cultures[a]		Virus yield (PFU/10^6 cells)[a]		RBE/liter of microcarrier culture	
	Microcarrier	Roller bottle	Microcarrier	Roller bottle	Cell basis	Virus basis
Sindbis-CEF[b]	2.4×10^8	5.0×10^7	3.1×10^9	5.2×10^9	48	29
Sindbis-CHO	3.0×10^8	1.3×10^8	1.8×10^9	1.2×10^9	23	35
MuLV-Cl-1[b]	3.6×10^8	4.1×10^8	2.1×10^5	5.7×10^5	9	3
SV-40-AGMK[c]	1.1×10^8	3.7×10^7	ND[d]	ND	30	
VSV-CEF[b]	2.0×10^8	8.2×10^7	1.0×10^8	3.4×10^8	24	7
Polio-WI-38	8.0×10^7	5.0×10^7	6.5×10^6	4.0×10^6	16	26
VSV-FS-4	1.8×10^8	4.0×10^7	ND	ND	45	

[a] Roller bottle area, 490 cm²; area of 100-ml microcarrier culture, approximately 3,000 cm². PFU, Plaque-forming units.

[b] Figures represent an average value obtained from at least two experiments.

[c] SV-40, Simian virus 40.

[d] ND, Not done.

Maximum yields obtained from roller bottles ranged from 2.1×10^7 in a volume of 25 ml to 5.2×10^7 when 150 ml of medium was used, or approximately 2.6×10^5 new cells produced per ml of whole medium. The microcarrier system gave a yield of 12.0×10^7 cells with 100 ml of medium and 21.0×10^7 from a volume of 150 ml, or approximately 11.0×10^5 new cells produced per ml of whole medium. The microcarrier system, then, yields approximately four times as many cells per milliliter of whole medium as a standard roller-bottle procedure.

DISCUSSION

It is obvious from the growth curves shown in Fig. 1 that a wide variation in both cell growth rates and maximum attainable cell density occur with our bead configuration. However, in all cases, cell attachment was good; cells remained attached, appeared healthy throughout the growth period, and formed complete monolayers on the microcarrier surfaces. There was no evidence of the toxicities and limited microcarrier concentrations experienced in earlier work with commercially available microcarrier sources (Levine et al., in press; 1, 9, 10, 13).

It must be borne in mind that our microcarriers were optimized for growth of one specific diploid fibroblast cell type (HEL-299, ATCC CLL137), and that no further attempts to optimize conditions for individual cell types were made. It is possible that, with microcarrier or environmental optimization, cell yields could be improved and lag times could be reduced. Nonetheless, even in the least attractive instance, i.e., Cl-1 cells, representing 9 RBE per liter of microcarrier culture (Table 1), advantages become apparent with scale-up: e.g., 100 liters replacing 900 roller bottles.

The variation in virus yields between microcarrier cultures and roller-bottle cultures (Table 1) also points out the need for optimizing conditions for each cell-virus system. VSV-CEF and MuLV-Cl-1 clearly gave the lowest virus yields on a per-cell basis in microcarrier cultures when compared with roller bottles, whereas the Sindbis virus-CHO and poliovirus-WI-38 systems showed the highest yields. No clear patterns can be established with existing data regarding the variation in virus yields seen in microcarrier-and roller-bottle-grown cells.

Only a small amount of published information is available regarding attempts to propagate viruses with microcarriers. Documentation that microcarrier-grown cultures provided a feasible way to mass-produce viruses was made by Van Hemert et al. (10) with rubella virus grown in BHK-21 cells and by van Wezel (11–13) with poliovirus grown in primary monkey kidney cells. Spier and Whiteside (9), using BHK cells to produce foot-and-mouth disease virus, actually quantitated cell growth and virus production on a per-cell basis, directly comparing yields from microcarrier cultures, Roux bottles, and regular suspension cultures. They observed good growth on diethylaminoethyl-Sephadex A50 beads if the beads were coated with serum, and they showed comparable virus yields with the three systems tested. Our studies comparing roller-bottle cultures with microcarrier cultures show that a wide variation in virus yields can be expected, depending upon the particular virus-cell combination used and the need in some instances for further optimization of conditions for cell and virus growth.

The decreased requirement for medium by CEF grown in microcarrier cultures in comparison with roller-bottle cultures was surprising, especially with regard to degree (fourfold greater yield per milliliter of medium with microcarrier cultures). No attempt was made in this study to determine the basis for the wide variation; however, it appears (through general observation and not testing) to be a general phenomenon applying to most if not all cell lines, regardless of the specific nutrient requirements of each. It is possible that the variation observed results simply from more efficient utilization of nutrients in a stirred suspension than in a static culture. It may, however, reflect real differences between the two systems (roller bottle and microcarrier) at the microenvironmental level. In any event, it appears that the use of microcarriers could result in substantial savings in the cost of culture media.

The desirability of having a workable system for growing anchorage-dependent cells in suspension culture has been discussed (Levine et al., in press; 1, 5, 9, 12). Advantages include a reduction in the cost of labor and materials, reduced risk of contamination, uniformity of product, and better control of environmental conditions. The potential value, of course, goes far beyond the production of cells and viruses, and includes the production of a wide variety of cell products, including interferon, enzymes, and hormones.

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