Mammalian Tissue Culture Growth, Viral Replication, and Cultivation Using Serum Replacement Factor (42945)

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Abstract. **Variability, cost, and availability of fetal bovine serum are in question; thus, we examined whether using a serum replacement factor in multiple mammalian tissue cultures could support not only cell growth, but also viral replication, expression, and retention of phenotypic markers. By using a serum replacement in defined media, we demonstrated multipassage cell growth of several different cell lines and viral cultivation and replication equivalent to fetal bovine serum.**
Moreover, by supplementing media with a serum replacement factor we observed at least a 28% financial savings. [P.S.E.B.M. 1989, Vol 192]

With the variability and supply issue of fetal bovine serum (FBS) in question, we examined whether using a serum replacement factor in multiple mammalian tissue cultures could support not only cell growth, but also viral replication, expression, and retention of phenotypic markers (1–3). Additionally, we examined the economics of serum replacement factor versus FBS since future prospects for purchasing FBS suggest low supply, high demand, and higher prices.

Only a few reports have demonstrated serum-free cultivation of lymphoid cells for virus production and cell lines or primary tissue for replication, cultivation, or viral susceptibility (4–7). In each case, chemically defined media selective or optimized for a specific cell line were used. Our goal was to find a serum replacement that would have the characteristics of a defined medium but, like FBS, would also stimulate various cell lines; i.e., cell yields and viabilities would be comparable to cells grown in FBS and would remain so after multiple passages. Moreover, the cells had to retain their susceptibility to viral infectivity, and viral yield had to be comparable to FBS-supplemented medium.

Our data demonstrate that using low protein serum replacement (LPSR) in medium gives multipassage cell growth, viral cultivation, and replication equivalent to FBS. Moreover, we found not only a financial savings of 28% but also a substantial savings in volume of FBS used (75%).

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Materials and Methods

Cell Cultures and Media. Human lung fibroblasts (HLF), MDCK, and Buffalo Green Monkey (BGM) cells were selected for this study since they are widely used and are susceptible to a variety of viruses. MDCK cells are kidney cells obtained from a female Cocker Spaniel and were purchased from American Type Culture Collection on its 57th passage. BGM, a continuous cell line, was derived from primary African Green Monkey (*Cercopithecus aethiops*) kidney and was obtained from Dr. Almen Barron of The State University of New York at Buffalo (8). HLF were developed in 1982 at the Cell Culture Activity, Biological Products Branch, OSS, CID at the Centers for Disease Control (CDC) in Atlanta by Dr. John Stuart, Virology Division at CDC from a tissue obtained from Brazil. These HLF cells are normal, diploid, female, human, and fibroblast-like. All three cell lines are anchorage dependent.

MDCK cells were grown (9) in Eagle's minimal essential medium (EMEM) with the addition of nonessential amino acids (0.1 m*M*), glutamine (2 m*M*), penicillin (200 units/ml), streptomycin (100 μg/ml), Hepes (10 m*M*) (Gibco Life Technologies, Grand Island, NY), insulin (0.13 units/ml), transferrin (5 μg/ml) (Sigma Chemical Co., St. Louis, MO), and selenous acid (5 ng/ml); all in final concentration. All medium contained either 7% FBS (Gibco, Grand Island, NY)

or 1.8% FBS and 1.1% LPSR-1[1] (Sigma Chemical Co.); the combination of LPSR-1 and FBS will be denoted as serum replacement (SR).

For the evaluation of LPSR-1 on MDCK cells, cells were passed 11 times (passage 58 to 69); the growth curve (Fig. 1A) was performed from passage 66. MDCK cells on passage 68 were seeded in 96-well plates (Costar, Cambridge, MA) and T-25 flasks (Corning, Corning, NY) at a concentration of 3×10^4 cells/well and 9×10^5 cells/ml, respectively. Half of the flasks and plates had FBS, the other half SR. The cells were incubated at 37°C under 5% CO_2 until a monolayer was obtained (approximately 3 days), the growth medium was removed, and the monolayer was washed three times with phosphate-buffered saline (10 m*M*, pH 7.2). Maintenance medium was added to cover the monolayer. Maintenance medium contains ingredients similar to those of growth medium but without FBS or SR. The medium is removed before inoculation with virus.

HLF were grown in EMEM with penicillin (100 units/ml), streptomycin (100 μg/ml), glutamine (2 m*M*), and Hepes (10 m*M*). HLF were either grown in 10% FBS or 2.5% FBS and 1.5% LPSR-1 (SR). The cells in this experiment were in their 20th passage from the original source when inoculated with virus and had been passed in the respective medium 10 times. Eight T-75 flasks were seeded with 5 million cells and allowed to grow to confluence, approximately 5 to 6 days.

BGM cells were grown in 500 ml of L-15 medium (Quality Biologicals, Gaithersburg, MD) and 500 ml of Hank's balanced salt solution containing 5 ml of glutamine (200 m*M*) (Gibco), 5 ml of amino acid solution 1, 5 ml of amino acid solution 2, 5 ml of vitamin solutions 1 and 2, 10 ml of Hepes (1 *M*) (Gibco), 5 ml of glucose solution, and 8 ml of 7% $NaCO_3$ with either 10% FBS or 2.5% FBS and 1.5% LPSR-1 (SR); designated BGM medium. The cell passages used for this study were from 223 to 245. The growth curve was performed from passage 234. The stocks for the experiments were inoculated at 2.5×10^4 cells/ml and seeded into T-75 flasks containing a volume of 20 ml. The tubes for the tests were inoculated at 1.5×10^5 cells/ml (1-ml volume/tube) and incubated at 37°C for 3 to 4 days. Before inoculation with the virus, the growth medium was removed.

Growth Curves. For all three cell lines the same basic procedure was follows: 32 T-25 flasks were inoculated with 1.5×10^5 cells of the corresponding cell line. Half of the flasks contained LPSR-1-supplemented medium and the other half had medium with FBS only (control). Cell counts were performed in duplicate approximately every 24 hr. The cell lines were in the following passages: MDCK, passage 66; HLF, passage 13, and BGM, passage 234.

Virus Growth. Influenza isolates were received from the State of Virginia Health Department and were supplied for the study by the Influenza Branch, Centers for Disease Control. Twelve different isolates were tested. A volume of 10 μl/well of undiluted and 10^{-1} dilution of influenza virus were inoculated onto MDCK cells (96-well plate), and 0.5 ml of undiluted sample was used to inoculate the T-25 flasks. The cells and virus were incubated for 2 hr at room temperature (RT), and maintenance medium containing trypsin (2 μg/ml) (Worthington Biochemicals Corp., Freehold, NJ) was added (10); 100 μl for 96-well plates and 4 ml for the T-25 flasks. The virus was allowed to grow for 3 days at 34°C, 5% CO_2.

After this incubation period, the following enzyme-linked immunosorbent assay (ELISA) was performed to detect infection of the cells by the influenza isolates. The monolayers were washed three times with phosphate-buffered saline (PBS), pH 7.2, the supernatant was aspirated, and the cell monolayer was fixed by adding 100 μl of 80% acetone (Fisher Scientific Co., Fairlawn, NJ) in PBS to each well and incubating the plates at 4°C for 15 min. The PBS was composed (in 1 liter) of 2.17 g of Na_2HPO_4, 0.02 g of NaH_2PO_4, 8.01 g of NaCl, and 0.20 g of KCl (all Fisher Scientific Co.). The plates were then allowed to dry under a chemical hood.

The plates were washed three times with PBS-0.05% Tween-20 (Fisher Biotech, Fairlawn, NJ) (PBS-Tween) and then tapped to remove excess fluid. To condition the monolayer and block possible sites of nonspecific binding, 50 μl of PBS-Tween with 1% fetal bovine serum were added and the plates incubated at RT for 30 min. Afterward, 50 μl of mouse monoclonal antibodies against Influenza type A-3 and B-4 (produced at CDC, Lawrenceville, GA facility) (both diluted 1/1000) were added appropriately to each well and the plates incubated for 30 min at RT. Then, these plates were washed three times with PBS-Tween as described previously. Goat anti-mouse peroxidase conjugate (100 μl; Tago, Inc., Burlingame, CA) diluted 1/2000 was then added to each well and incubated for 30 min at RT and then washed as described previously. The substrate buffer solution was prepared by making a 40 mg/100 ml (w/v) solution of *o*-phenylenediamine in citrate-phosphate buffer (pH 5.0, 486 ml of 0.1 *M* citric acid, 500 ml of 0.2 *M* sodium phosphate, adjust pH to 5.00 and volume adjusted to 1000 ml). Of this solution, 100 μl/well were added and incubated for approximately 20 min at RT. Color development was stopped by adding 50 μl/well of 1 *M* H_2SO_4 (Fisher Scientific Co.). Absorbance was read in a Microplate Reader (model

[1] LPSR-1 is a mixture of growth-enhancing components and carrier proteins. This includes albumin, transferrin, insulin, other hormones, vitamins, attachment factors, soybean trypsin inhibitor, and other growth factors. The actual concentration of its components is proprietary information of the manufacturer. LPSR-1 may be used to replace serum or reduce its concentration in cell growth media (Sigma Chemical Co., Cell Culture News Bulletin, Vol. 4 (1), April 1988).

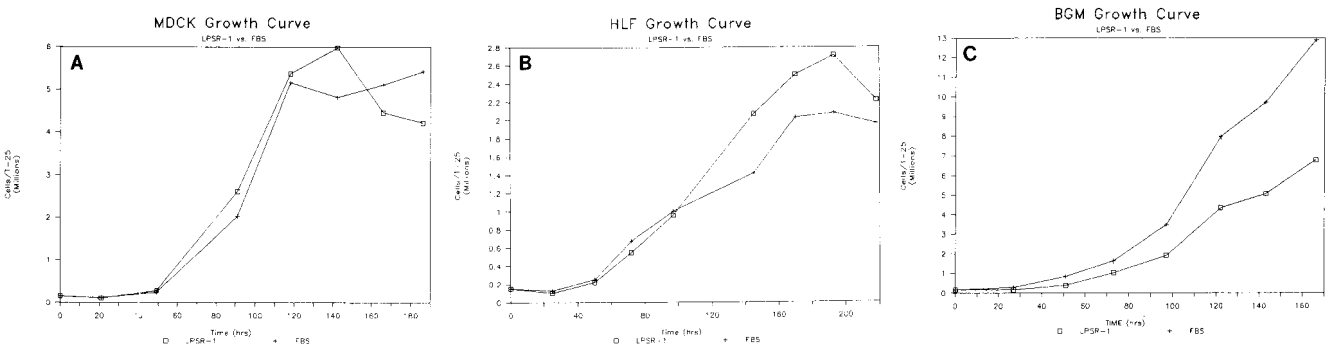

Figure 1. Growth comparisons on cell lines supplemented with LPSR-1 to cells grown in FBS only (control): (A) MDCK cells, passage 66. (B) HLF cells, passage 13. (C) BGM cells, passage 233.

MR600; Dynatec Laboratories Inc., Alexandria, VA). The settings used were: wavelength R = 5, wavelength T; eq 3, calibrated o.d. × 1.00 and threshold >1.00.

The cell control consisted of uninfected cells which underwent the ELISA procedure. The lowest reading blank well was used to blank the instrument. The cutoff point used for these experiments was the cell control mean plus four times the standard deviation.

HLF cells were used to examine cytomegalovirus (CMV) serotype AD169 growth. After removing the media from HLF confluent cells, 20 μl of CMV were added. The virus was allowed to adsorb for 1 hr at 37°C. Afterward, the monolayer was covered with 25 ml of Medium 199 with either 2% FBS or 1.0% FBS and 0.5% LPSR-1 and incubated for approximately 14 days or until cytopathic effects (CPE) developed 3–4+.

For BGM cells, 10 different virus types were tested: Echo virus of the serotypes 16, 17, 21, 23, 30 (Frater), 30 (Giles), and 33; also, polio virus serotype 3 (Sabin), REO serotype 1, and coxachie serotype B-2. Two maintenance media were used to grow the virus; 2% FBS or 0.4% LPSR-1 plus 1% FBS, both in EMEM. To each tube, 100 μl of the virus sample were inoculated and the virus was allowed to grow for 8 days. Tubes were observed daily for CPE development.

Virus Titration. Influenza virus was titrated by collecting the spent media from the T-25 flasks in which the virus isolates were grown. Ten-fold dilutions (undiluted to 10^{-7}) of this medium were prepared and 10 μl/well of each were added to 96-well plates previously inoculated with MDCK cells grown in either FBS or SR. The virus was allowed to adsorb for 1 hr at RT. Afterward, 100 μl of trypsin containing maintenance medium were added to the plates and allowed to incubate for 3 days at 34°C, 5% CO_2. An ELISA was performed to detect virus infection.

For titration of CMV, the supernatant was removed from flasks and diluted 10-fold up to 10^{-8}; 1 ml of each dilution was inoculated into T-25 flasks containing HLF monolayers and allowed to adsorb for 1 hour at 37°C, after which the inoculum was removed. The monolayer was covered with Medium 199 with either 2% FBS or 0.5% LPSR-1. The virus was allowed to grow for 1 to 2 weeks. When CPE had progressed, Giemsa stain was added to the monolayer, and plaques were counted.

To titrate viral growth in BGM cells, we used tubes as previously described for viral growth using BGM cells. Supernatant of each viral isolate was collected from the viral growth assay and tested for its capacity to reinfect BGM cells. The supernatant was diluted 10-fold up to 10^{-7}, and tubes containing BGM cell monolayer were inoculated and incubated. The tubes were checked daily for 8 days, and presence or absence of cytopathic effects was observed.

Results

In Figure 1, cell yields are compared when grown in SR to FBS. Through Day 5, the curves were comparable. After 120 hr, cell growth with SR appeared higher for cell line HLF (Fig. 1B) and lower for cell line BGM (Fig. 1C) when compared with FBS only. In all cases, cell viability was always greater than 95% in SR (data not shown). All growth curves are representative of three individual experiments performed in duplicate.

Viral growth was examined for each cell line. For MDCK cells, 12 isolates of influenza virus were compared. In all cases, the isolates which were Type A-3 as shown by an ELISA (Table I), replicated equally well in SR or FBS only (data not shown). HLF cells were used to assay CMV virus growth. Similar microscopic results of 3+ CPEs were observed. Titration results suggest (Table II) that SR is equivalent and competitive in supporting virus growth to FBS. BGM cells were inoculated with multiple viruses, and in all cases using different viral passages (Table III) the CPEs were equivalent (Day 2 through Day 8).

When MDCK cells were used to examine influenza virus titration studies in the presence of either SR or FBS (Table I), the SR was equivalent or better (isolates 1–7) than FBS. When BGM cells were examined (Fig. 2), no significant differences were noted.

Discussion

Cells *in vivo* exist in and react to a complex set of influences. If a cell is to survive *in vitro*, it must be

Table I. Titration of Influenza Virus Isolates (Type A-3) Using MDCK Cells Grown in LPSR-1 and FBS[a]

Isolates	Dilutions				
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}
1 LPSR-1	+	+	+	+	–
FBS	+	+	+	–	–
2 LPSR-1	+	+	+	–	–
FBS	+	+	–	–	–
3 LPSR-1	+	+	+	–	–
FBS	+	+	+	–	–
4 LPSR-1	+	+	–	–	–
FBS	+	+	–	–	–
5 LPSR-1	+	+	+	–	–
FBS	+	+	–	–	–
6 LPSR-1	+	+	–	–	–
FBS	+	+	–	–	–
7 LPSR-1	+	+	–	–	–
FBS	+	–	–	–	–
8 LPSR-1	+	–	–	–	–
FBS	+	+	–	–	–

[a] By ELISA using an anti-Type A-3 antibody.

Table II. CMV Virus Growth in HLF Cells

Growth conditions[a]	Plaques/ml (log)	
	Experiment 1	Experiment 2
FBS[b]/FBS/FBS	4.34 ± 0.15	5.14 ± 0.16
FBS/FBS/SR	3.91 ± 0.03	5.37 ± 0.17
FBS/SR/FBS	5.49 ± 0.06	3.53 ± 0.18
FBS/SR/SR	4.31 ± 0.08	3.99 ± 0.09
SR/FBS/FBS	6.31 ± 0.08	5.24 ± 0.23
SR/FBS/SR	6.19 ± 0.08	5.86 ± 0.20
SR/SR/FBS	5.76 ± 0.37	4.15 ± 0.08
SR/SR/SR	5.11 ± 0.28	4.74 ± 0.19

[a] Cell growth/viral growth/virus titration.
[b] FBS, EMEM with 10% FBS concentration; SR, EMEM with 1.5% LPSR-1, 2.5% FBS for cell growth. For virus growth and titrations, FBS = 2% FBS, SR = 0.5% LPSR-1, both in EMEM.

Table III. Virus Growth in BGM Cells

Virus type	Virus passage	CPE		
		FBS/FBS[a,b]	SR/FBS	SR/SR
Polio 3 Sabin	1	Day 2, 4+	Day 2, 4+	Day 2, 4+
Coxachie B-2	1	Day 2, 4+	Day 2, 4+	Day 2, 4+
Echo 23	2	Day 8, 3+	Day 8, 3+	Day 8, 2+
Echo 33	1	Day 5, 4+	Day 5, 4+	Day 5, 4+
Polio 3 Sabin	4	Day 3, 4+	Day 3, 4+	Day 3, 4+
Coxachie B-2	4	Day 3, 4+	Day 3, 4+	Day 3, 4+

[a] Cell growth/virus growth.
[b] FBS, BGM medium with 10% FBS; SR, BGM medium with 1.5% LPSR-1 and 2.5% FBS for cell growth. For virus growth, FBS = 2% FBS, SR = 1% FBS, and 0.5% LPSR-1.

placed in a culture system where the medium resembles the *in vivo* environment. In the past, cells *in vitro* not only required basal nutrients, but also poorly defined biologic fluids and/or extracts such as fetal bovine serum. Variability, cost, and availability of these biologic fluids made us examine whether a serum replacement factor could support *in vitro* cell growth as well as *in vitro* viral replication, expression, and retention of phenotypic markers in established mammalian cell lines. Our data suggest that there are serum replacement factors that have the characteristics of a defined medium. Like FBS, these factors stimulate various cells and allow for cell growth.

Table IV. Cost Comparison per Liter of Medium

	10% FBS	2.5% FBS, 1.5% LPSR-1[a]
Medium	$8.00	$8.00
Supplements[b]	5.00	5.00
FBS	40.00	10.00
LSPR-1	0.00	15.00
Totals	$53.00	$38.00

[a] The cost of LPSR-1 is $1.00/ml compared with $0.40/ml for FBS assuming that LPSR-1 gives five times the growth-supporting equivalent as FBS (per volume, Sigma Chemical Co.). The cost for LPSR-1 is $0.20/ml.
[b] Glutamine, penicillin/streptomycin, and Hepes.

We compared several cell lines for cell growth and viability (Fig. 1). Although our data demonstrate comparable or better growth with SR, we have also observed less efficient growth (Fig. 1C). Adaptability and passage of cells must be examined on an individual cell line basis.

Serum replacement can induce morphologic changes as we have observed with the mouse cell line McCoy (data not shown). Whether these changes represent a difference in ability to retain phenotypic markers and support of an intracellular parasite is under study.

We conclude that SR was comparable to FBS in cell growth, viral infectivity, and yield as well as expression (Tables 1–3). We hope to establish a serum replacement system for cell growth and virus propagation that will allow for the production of viral vaccine. At present, we are studying the use of serum replacements with cell lines approved by the Food and Drug Administration for vaccine production. Additionally, since some clinical isolates of virus require longer incubation periods, we are presently examining SR-supplemented media for use in a clinical setting with human lung fibroblast cell cultures. Preliminary data suggest that SR supports long-term culture similar to FBS. Again, we stress that the adaptability and passage of cells must be examined on individual cell line basis.

We examined not only the economic advantages of a serum replacement factor (28%), but also the savings in volume of FBS used (75%) (Table IV). We also found the need for storage space considerably less since LPSR-1 comes in lyophilized form. These factors along with comparable cell growth and viral replication

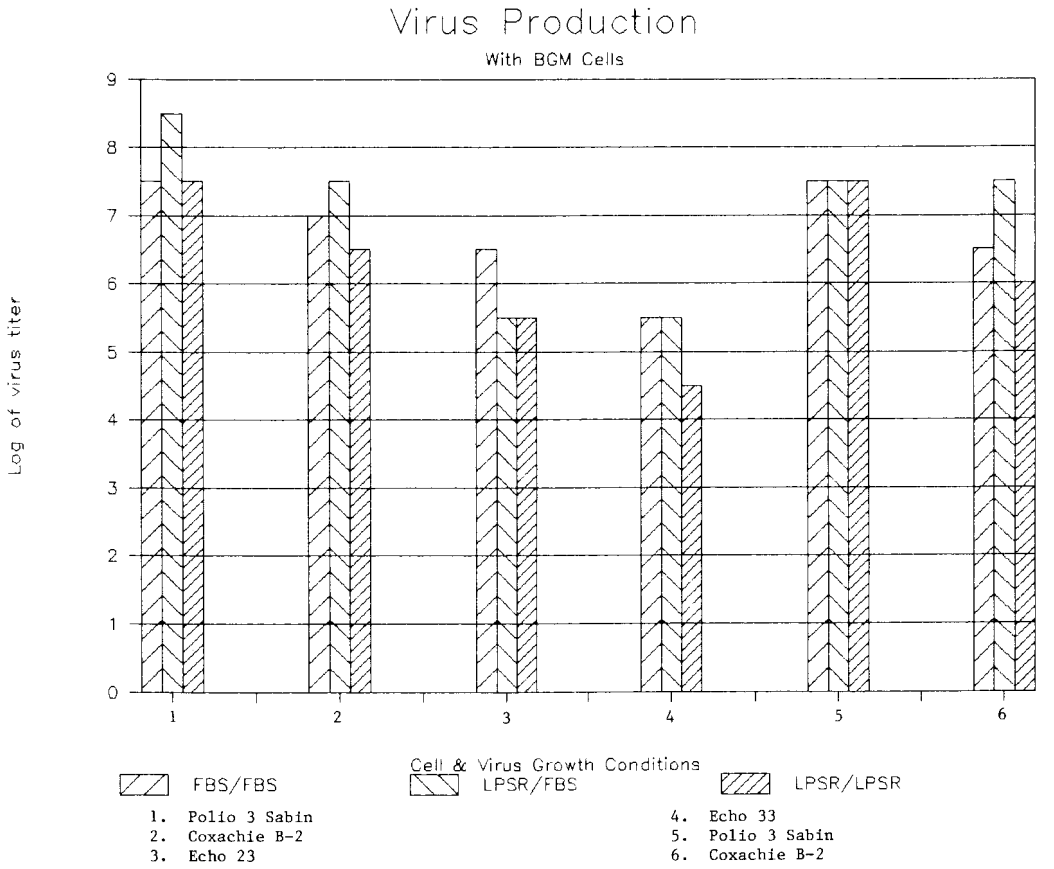

Figure 2. Replication of different virus types in BGM cells. Cytopathic effects were used as evidence of virus growth.

indicate that further analysis and use of SR in large-scale bioreactors for mammalian cell tissue culture are warranted.

Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

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