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CHEMICALLY DEFINED SERUM-FREE MEDIA FOR THE CULTIVATION OF PRIMARY CELLS AND THEIR SUSCEPTIBILITY TO VIRUSES

S. A. WEISS,[1] T. L. LESTER, S. S. KALTER, AND R. L. HEBERLING

Southwest Foundation for Research and Education, San Antonio, Texas 78284

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SUMMARY

Chemically defined media SFRE-199-1 for the growth and SFRE-199-2 for the maintenance of primary baboon kidney (Bak) cell cultures were formulated by supplementing medium M199 with insulin, sodium pyruvate, zinc sulfate, and increasing arginine-HCl, cysteine, cystine, L-glutamine, L-glutamic acid, glycine, histidine, tyrosine, and glucose to maximally active nontoxic concentrations. For prolonged maintenance of the cells, physiological pH control, and blocking of excessive lactic acid accumulation in the spent medium of the cell cultures, it was necessary to supplement the medium containing Earle's balanced salts with D-(+) galactose.

The cells grew and were maintained equally well on glass or polystyrene surfaces. Selenium, when added to growth medium or substituted for insulin and zinc sulfate, did not stimulate cell growth. Electron microscopy showed that numerous dense particles, approximately 250 to 400 Å in diameter, with the appearance of glycogen, were found throughout the cytoplasm in the cells grown in SFRE-199-1 and maintained in SFRE-199-2. Echovirus types 1 to 3, poliovirus types 1 to 3, coxsackievirus types B2, B4, B5, *Herpesvirus hominis* type 1, simian herpesvirus *H. simiae* and SA8, and simian adenovirus SV34 when titrated in primary Bak cells and grown and maintained in SFRE-199-1 and 2, respectively, developed titers comparable to those obtained in conventionally grown and maintained cells.

Key words: chemically defined medium; primary baboon kidney cells (Bak); selenium; protamine zinc insulin (PZI); galactose; amino acids; zinc sulfate.

INTRODUCTION

Optimal growth of mammalian cells in tissue culture is routinely obtained with medium supplemented with whole animal or human serum. Various macromolecular components present in whole mammalian serum have been shown to be necessary for cellular multiplication, but the role and mechanism of action of these factors have not been definitively ascertained (1). Wide differences in chemical and hormonal parameters found in the various lots of sera used may explain some of the variability and lack of reproducibility in tissue culture studies (2,3). Many biological products and reagents, including interferon and live virus vaccines, are produced utilizing tissue and cell cultures supplemented with animal or human serum (4). Furthermore, cell cultures are also being increasingly utilized in studies of cancer, cytotoxicity, nutrition, cellular differentiation, and other biological phenomena.

A number of investigators have shown that animal sera and their corresponding donors may be contaminated with mycoplasmas, bacteriophages, and a variety of animal viruses, which could eventually find their way into the final product (5-7).

Nutritional and growth requirements of the various mammalian cells in culture have been the subject of several classical studies (8-11). Established cell lines and strains derived from them have been used extensively in establishing nutritional requirements, but few data are available where a chemically defined medium is employed for the growth of primary cell cultures. Various

[1]To whom orders for reprints should be addressed at Department of Microbiology and Infectious Diseases, Southwest Foundation for Research and Education, P.O. Box 28147, San Antonio, Texas 78284.

investigators have reported on serum substitutes for mammalian cells; these include whole and fractionated peptone dialysates, lactalbumin hydrolysate, blood fractions, trace metals, ovomucoid, soybean trypsin inhibitors, hormones and corticoid steroids or combinations of the above (11-13). Ham elaborated on the nutritional requirements for primary cultures and the need for this information in biological research (14). This need is emphasized in the development of medium 199 (M199) by Morgan et al. (8,15). It should be noted that M199 was originally developed for use in studies of chick embryo muscle fibroblast nutrition, without the addition of serum or other uncharacterized materials. Eagle, in his formulation of minimal essential medium (MEM), defined the amino acid requirements for monkey kidney cells in primary culture and their metabolism in mammalian cell cultures (16,17).

The wide application of M199 (8) and Eagle's MEM for the growth of a variety of mammalian cell cultures has been taken into consideration, and these media have been used as the basis on which a chemically defined medium was formulated for the growth and maintenance of primary baboon kidney cell cultures.

Materials and Methods

Growth medium (SFRE-199-1). The formulation of chemically defined media for growth and maintenance of primary baboon kidney (Bak) cell is presented in Table 1. Growth medium, SFRE-199-1, differs from M199 (8) by the increased concentration of eight amino acids and glucose, the addition of zinc sulfate ($ZnSO_4 \cdot 7H_2O$), sodium pyruvate, and protamine zinc insulin suspension (USP). In addition, there are decreased concentrations of ferric nitrate [$Fe(NO_3)_3 \cdot 9H_2O$], xanthine, and sodium bicarbonate ($NaHCO_3$). Growth medium contains Hanks' balanced salt solution (HBSS) (18).

Maintenance medium (SFRE-199-2). Maintenance chemically defined medium differs from the growth SFRE-199-1, in that it contains Earle's balanced salts (EBSS) (19) with the addition of D-(+) galactose. For the growth and maintenance of Bak cells, conventional procedures that have been described elsewhere were followed (20).

Preparation of media. Initially, experimental media was prepared in 2-l volumes, but thereafter those media that supported the growth of primary Bak to full confluency were prepared in lots of 20 to 40 liters. Basic powdered tissue culture medium, M199, was purchased from a commercial source (K C Biological, Lenexa, KS, with EBSS and HBSS, respectively).

Ingredients under investigation for their optimum nutritionally active, nontoxic concentrations were omitted from the basic powdered media by the manufacturer. After establishing the optimum concentrations of these ingredients (see Table 1), the manufacturer was instructed to prepare the complete formulation of the media, omitting only insulin and sodium bicarbonate. The amount of powdered medium specified by the supplier was weighed out in our laboratory and transferred into 40-liter stainless steel tanks (Irvine Scientific Co., Irvine, CA) containing the appropriate volume of deionized and subsequently distilled water. After the powder was completely dissolved, the required amount of sodium bicarbonate was added while being stirred on a nonheating stirrer with a 2-inch egg-shaped magnetic bar. Prior to sterilization, the pH of the media for growth and maintenance was adjusted to 6.9 to 7.0 and 7.5 to 7.6, respectively. The media were sterilized by serial membrane filtration in a Millipore filter holder (Millipore Corp., Bedford, MA). Briefly, the filtration system was assembled as described: A 257-mm Millipore, Dacron woven space holder was placed on the top of the filter support screen in the holder and this was followed by a 293-mm Gelman TCM-200 Metricel membrane filter with 0.2-μm porosity, and a 293-mm Gelman TCM-450 Metricel membrane filter with 0.45-μm porosity (Gelman Sciences, Ann Arbor, MI). Finally, a Millipore AP20 prefilter was layered on top of the assembly.

Silastic medical grade silicone tubing, 1/4 inch i.d. × 1/2 inch o.d., manufactured by Dow Corning Corporation (Dow Corning Corp., Midland, MI), was used for connection of the filter holder to the dispensing bell and pressurized media tank. The complete filtering apparatus was sterilized by steam under pressure at 121° C for 1 hr using a dry cycle on a Castle Powerclave sterilizer (Castle Co., Rochester, NY). After filtration, all lots of media were tested for sterility and then stored at 4° C.

Prior to its addition to media, insulin was prediluted in M199 with HBSS instead of in 0.15 *M* of NaCl (21). Procedures for the addition of insulin to the cell suspension have been described previously (22).

Briefly, the protective cap of the vial containing 10 ml of protamine zinc insulin suspension (PZI) was removed and the stopper cleaned with cotton

TABLE 1

FORMULATION OF CHEMICALLY DEFINED MEDIUM SFRE-199-1 FOR GROWTH AND MEDIUM SFRE-199-2 FOR THE MAINTENANCE OF THE PRIMARY CELLS[a]

Components	Medium 199[b]	SFRE-199-1 and SFRE-199-2
	mg/l	*mg/l*
Amino acids		
L-Alanine	25.0	25.0
L-Arginine HCl	70.0	150.0
L-Aspartic acid	30.0	30.0
L-Cysteine (free base)	0.1	4.0
L-Cystine (disodium salt)	20.0	40.0
L-Glutamic acid	67.0	75.0
L-Glutamine	100.0	300.0
L-Glycine	50.0	100.0
L-Histidine HCl·H_2O	20.0	40.0
Hydroxy-L-proline	10.0	10.0
L-Isoleucine	20.0	20.0
L-Leucine	60.0	60.0
L-Lysine HCl	70.0	70.0
L-Methionine	15.0	15.0
L-Phenylalanine	25.0	25.0
L-Proline	40.0	40.0
L-Serine	25.0	25.0
L-Threonine	30.0	30.0
L-Tryptophan	10.0	10.0
L-Tyrosine	40.0	80.0
L-Valine	25.0	25.0
Vitamins		
Ascorbic acid	0.050	0.050
D-Biotin	0.010	0.010
Calciferol	0.100	0.100
D-Ca pantothenate	0.010	0.010
Choline chloride	0.500	0.500
Folic acid	0.010	0.010
i-Inositol	0.050	0.050
Menadione	0.010	0.010
Nicotinamide	0.025	0.025
Nicotinic acid	0.025	0.025
p-Aminobenzoic acid	0.050	0.050
Pyridoxal HCl	0.025	0.025
Pyridoxine HCl	0.025	0.025
Riboflavin	0.010	0.010
Thiamine HCl	0.010	0.010
DL-αTocopherolphosphate (Na_2)	0.010	0.010
Vitamin A (acetate)	0.140	0.140
Other components		
Adenine sulfate	10.00	10.00
Adenosine-5′-triphosphate disodium, tetrahydrate (ATP)	1.08	1.00
Cholesterol	0.20	0.20
Deoxyribose	0.50	0.50
D-Glucose	1,000.00	2,000.00
D-(+) Galactose	—	1,000.00
$Fe(NO_3)_3 \cdot 9H_2O$	0.72	0.3
Glutathione	0.05	0.05
Guanine HCl	0.30	0.30
Hypoxanthine	0.30	0.30
Insulin[c]	—	0.15 IU/ml
Ribose	0.50	0.50
Phenol Red	10.00	10.00
Sodium acetate	50.00	50.00
Sodium pyruvate	—	150.00
Thymine	0.30	0.30
Tween 80[d]	5.00	5.00
Uracil	0.30	0.30
Xanthine	0.34	0.30
$ZnSO_4 \cdot 7H_2O$	—	0.10
Inorganic salts	Hanks' salt	Hanks' salt
$CaCl_2$ (anhydrous)	140.0	140.0
KCl	400.0	400.0
KH_2PO_4	60.0	60.0
$MgSO_4O$ (anhydrous)	97.70	97.70
NaCl	8,000.00	8,000.00
$NaHCO_3$	1,400.00	350.00
Na_2HPO_4 (anhydrous)	48.0	48.0
	Earle's salt	*Earle's salt*
$CaCl_2$ (anhydrous)	200.0	200.0
KCl	400.0	400.0
$MgSO_4$ (anhydrous)	97.7	97.7
NaCl	6,800.00	6,800.00
$NaHCO_3$	2,200.0	2,200.0
$NaH_2PO_4 \cdot H_2O$	140.0	140.0

[a] Prior to filtration, pH of CDM varied from 6.5 to 6.6 and was adjusted to 6.9 to 7.0 for growth; for maintenance, pH was brought up to 7.5 to 7.6 by the addition of 2.5 *N* NaOH. Underlined are the maximum active nontoxic concentrations of the nutrients in CDM.

[b] (8,15).

[c] Protamine zinc insulin suspension, USP, Eli Lilly and Co., Indianapolis, IN.

[d] Trademark of ICI Americas Incorporated.

dipped in 70% ethanol. With the plastic sterile disposable 10-ml syringe, equipped with a sterile disposable 20 G 1-inch needle, PZI was withdrawn from the vial and transferred as quickly as possible into a 250-ml Erlenmeyer flask containing 90 ml M-199 with HBSS. A steam-sterilized magnetic bar, 1½ inch long, was also placed in the Erlenmeyer flask.

The suspension of PZI was mixed and, with the same syringe, 5 ml of the suspension of PZI in M-199 was withdrawn and injected back into the vial. Without removing the needle from the rubber cap, the contents of the vial were withdrawn back into the barrel of the syringe, then injected back into the vial several times to rinse and remove all the PZI suspension from the vial. The contents from the vial were quickly transferred into an Erlenmeyer flask containing PZI in M-199, which was then placed on the nonheating magnetic stirrer set at moderate speed at room temperature. Stirring was continued for 1 to 1½ hr prior to adding cell bulk suspension. Final concentration of PZI in cell bulk suspension is adjusted to 0.15 IU/ml. When the cell density of the bulk cell suspension in the medium was adjusted to 1.5×10^5 cells/ml, prediluted PZI was added to the bulk cell suspension at a final concentration of 0.15 IU/ml. At this point, the final bulk cell suspension was stirred for 20 min before planting into appropriate vessels. Protamine zinc insulin with a glucagon content of 0.03% v/v as determined by radioimmunoassay was used in all the experiments reported in our studies (23). The zinc content varied between 0.01 and 0.04 mg/100 U of insulin and the protamine content was 1.15 mg/100 U of insulin.

Dissociation of the primary baboon kidney cells. Methods previously described by Hull et al. (24) have been followed for the preparation of primary Bak with the following modifications. Instead of a conventional trypsin solution, a primary dispersing agent (PDA) was employed throughout these studies. Primary dispersing agent contains the following concentration of each component per liter of distilled water: 2 g trypsin (1:250), 0.05 g enzyme concentrate 1917, 8 g NaCl, 0.383 g KCl, 0.1 g $Na_2HPO_4 \cdot 7H_2O$, 1.0 g dextrose, and 3.0 g Tris. The final pH was adjusted to 7.6 prior to sterilization by filtration. Primary dispersing agent was prepared in 20- to 40-liter lots and after filtration stored at −15° C for up to 18 months. In general, PDA treatment produced a preponderance of single dispersed cells.

Enzyme concentrate 1917 (Reheis Chemical Co., Kankakee, IL) is a purified, high potency proteolytic enzyme mixture of porcine pancreas origin. It principally contains trypsin and chymotrypsin, with lesser components of elastase, peptidases, and esterases.

Dissociation of Bak cells was achieved at 4° C overnight (15 to 16 hr) by stirring the kidney fragments in a 500-ml flask (Bellco Glass, Inc., Vineland, NJ) at 130 rpm with a 2-inch magnetic bar and a Bellco nonheating magnetic stirrer. Trypsinized Bak cells were filtered through a funnel equipped with a 60-mesh screen. The funnels and screens were fabricated from 316 stainless steel.

After three consecutive washes with HBSS and centrifugation at 1000 rpm for 5 min, the cell pellet was resuspended in 50 ml of HBSS and filtered again. The filter screen was rinsed with 50 ml of HBSS to ensure removal of the cells, which were then diluted for viable cell counting employing Trypan Blue dye exclusion test. Appropriate cell suspensions were prepared in the respective media for planting. Employing the above modification of the standard procedure, we have consistently obtained a total of 7×10^8 viable cells from a pair of kidneys from a 2-year-old animal weighing 5 to 7 kg.

Culture procedure. In all the experiments involving nutritional studies with serum-free chemically defined medium, as well as conventional controls, cell inocula consisted of 1.5×10^5 cells/ml or 6×10^4 cells/cm² of surface area in the growth vessels. These vessels were inoculated with cells suspended in the various experimental growth media and then incubated for up to 96 hr at 36.5° C. After being changed to maintenance medium, cells were reincubated for an additional 72 hr. Cells grown and maintained in the conventional medium reached confluency by the 6th or 7th day postplanting. At this time, media variations in which the cells reached full confluency were selected for further studies. Monolayer cultures were grown in polystyrene Corning tissue culture flasks (25 cm², 75 cm², and 150 cm², Corning Glass Works, Corning, NY). Replicate cell cultures were removed at different intervals postplanting for serial subculturing, histological staining, and electron microscopy studies.

For the study of attachment, growth, and maintenance on a glass surface, cell suspensions in various growth media were planted in Brockway 32-oz Rx Sani-Glas bottles (130 cm², Brockway Glass Co., Parkersburg, WV) or Brockway 125- × 16-mm tubes with screw caps. Cell mono-

layers in tubes were used for virus titrations. For histological staining and subsequent examination, the cells were planted in Brockway screw-cap 125 × 16-mm tubes each containing a Bellco 9 × 12-mm micro cover glass treated for tissue culture application (25).

During nutritional studies, Bak cell cultures were examined microscopically every day for cell attachment, morphological changes, rate of confluency, and cytotoxicity according to criteria described elsewhere (26).

For secondary and subsequent passaging, cells were harvested from monolayer cultures by a mild and rapid treatment as follows. Growth medium was removed from confluent monolayers in Corning T-75 culture flasks and the cell sheet washed with 10 ml of an enzyme concentrate-ethylenediaminetetraacetic acid solution (EC-EDTA) warmed to 37° C in a water bath. Enzyme concentrate-ethylenediaminetetraacetic acid solution is composed of 0.3 g enzyme concentrate (Reheis, 0.2 g ethylenediaminetetraacetic acid (Fisher Scientific Co., Pittsburgh, PA), 8 g NaCl, 0.2 g KCl, 1 g glucose, and 0.02 g Phenol Red (GIBCO, Grand Island, NY) in deionized and subsequently distilled water brought to a total volume of 1 liter. Prior to sterilization by filtration, the pH was adjusted to 7.6. By gently rocking the flask, even exposure of the cell sheet to EC-EDTA was achieved. The solution was then decanted. The monolayer, wetted only by a thin film of EC-EDTA that remained in contact with the cell sheet, was allowed to stand at 36.5° C for 1 to 2 min or until the cells came off. Five milliliters of chemically defined medium (SFRE-199-1) were added to each T-75 flask and the vessel was shaken; this was followed by gentle pipetting to insure an even dispersion of the cells. An appropriate sample from the resultant suspension was removed for a viable cell count (27).

Measurement of cell growth response. On the 7th day postplanting, when the cells grown in lactalbumin hydrolysate in HBSS (HLH) with 5% fetal bovine serum (FBS) (conventional control) usually reached confluency, microscopic reading of the experimental variations along with the controls were recorded. Replicates from each experimental variation were subdivided into several groups. Consequently, six 75-cm^2 culture flasks from each group were taken for scoring and staining of the cell sheets (28,29); they were trypsinized for cell counts and further subculturing, electron microscopy, and virological studies. The remaining cultures were left incubating on maintenance media with continued observation until 21 days postplanting. In these cultures, the medium was replaced every 5 days.

An evaluated medium was considered acceptable if the cell sheet confluency rate, the absence of toxicity, and the cell yields were comparable to conventional control cultures.

Virus susceptibility studies. Viruses used in these studies were obtained from the NIH/WHO Collaborating Center for Reference and Research in Simian Viruses located at the Southwest Foundation for Research and Education (30).

Prior to titration, enteroviruses were passaged three times in Bak cells that were grown and maintained in SFRE-199-1 and SFRE-199-2, respectively. The DNA viruses were titrated without serial passaging. Cell cultures in tubes were inoculated with 0.1 ml of undiluted virus and observed for cytopathic effects (CPE) (31). If 3+ to 4+ CPE was observable before 14 days postinoculation, culture tubes were frozen at −20° C before subsequent virus passage. Virus fluids from the third passage were titrated on Bak in chemically defined medium and titers were calculated (and expressed as $\log_{10}$ $TCID_{50}$ per 0.1 ml) according to the method of Reed and Muench (32). At the various times postinfection, when 2+ CPE was noted, cover slips from the culture tubes were removed, fixed, stained, and examined for viral inclusion bodies (33).

Biochemical analysis. Cell-free protein in the spent maintenance SFRE-199-2 medium was measured by the method of Oyama and Eagle (34). For an analysis of the zinc and selenium in tissue culture reagents, atomic absorption spectrophotometry techniques were used (35). Levels of lactic acid in the culture medium were assayed enzymatically with Sigma β-nicotinamide adenine dinucleotide reaction kits (Sigma Chemical Co., St. Louis, MO) (36).

RESULTS

In the formulations of SFRE-199-1 and SFRE-199-2 media the concentration of arginine, cysteine, cystine, glutamine, glutamic acid, glycine, histidine, and tyrosine had to be increased to concentrations higher than in the standard formulation of M199 to obtain maximum cell growth (Table 1). The formulation of SFRE-199-1 medium for growth and SFRE-199-2 medium for maintenance is presented in Table 1. Maximum active nontoxic concentrations of the components in the media are underlined in Table 1.

TABLE 2

EFFECT OF SFRE-199-1 AND SFRE-199-2 ON THE RATE OF CONFLUENCY, CELL YIELD, AND PROTEIN CONTENT IN THE SPENT MEDIUM OF PRIMARY BAK CELLS[a]

Medium	Growth Rate of Cells on Days Indicated[b]						Cell Yield per Vessel on the 7th Day Postplanting[c]	Protein Content in the Spent Medium on Days Postconfluency	
	2	3	4	5	6	7		1	4
								$\mu g/ml$	
HLH + 5% FBS	±	++	++	+++	++++	++++	1.9×10^7	1400	1413
M199 + 3% FBS	+	++	++	+++	++++	++++	1.8×10^7	1200	1375
M199 + insulin	±	±	+	++	++	++	6.5×10^6	900	750
M199 + insulin + $ZnSO_4 \cdot 7H_2O$	±	±	+	++	+++	+++	7.8×10^6	970	800
SFRE-199-1	+	++	++	+++	++++	++++	1.9×10^7	1350	1200
SFRE-199-1 − insulin	±	+	+	++	++	++	6.8×10^6	1050	800
SFRE-199-1 − $ZnSO_4 \cdot 7H_2O$	±	+	+	++	++	++	6.4×10^6	1025	760
SFRE-199-1 + 15 n*M* Se	±	±	+	++	+++	++(t)	5.8×10^6	980	650
SFRE-199-1 + 30 n*M* Se	±	±	+	++	++(t)	++(t)	5.1×10^6	970	475

[a] Cell inocula consisted of 1.5×10^5 cells/ml of medium.
[b] Method of scoring of the cell growth: ± = <25% cell sheet; + = 25% Cell sheet; ++ = 50% cell sheet; +++ = 75% cell sheet; ++++ = 100% confluent monolayer; t = toxic trace.
[c] Average calculated from six T-75 culture flasks.

The growth response, as determined by the rate of achieving confluency, cell yield, and protein content in spent medium, is summarized in Table 2. Cells grown in the medium free of serum had the same growth rate, cell yield, and protein content as cells grown in M199 supplemented with 3% FBS or HLH with 5% FBS. Monolayers reached full confluency on the 6th day postplanting with all three media. Primary Bak cells grown in SFRE-199-1 attained a population doubling time (PDT) of approximately 34.2 hr, which was the same as for cells grown in HLH with 5% FBS. Cells grown in M199 with 3% FBS had a PDT of 36 hr. Chemically defined medium also supported the growth of Bak cells in the 3rd passage as well as cells in the other two media (Table 3). The population doubling time of these cells was 26 hr, or 8.2 hr shorter than for primary Bak cells grown in chemically defined medium.

When M199 was supplemented with insulin alone, monolayers were not confluent after 7 days in culture and the PDT was 102 hr (Table 2). When zinc was added to M199 containing insulin, the growth rate was increased and the PDT was 83.2 hr, but the cell sheet reached only 75% confluency in 7 days. In the experiments in which insulin was omitted from SFRE-199-1, cell sheets did not reach confluency during 21 days of observation, and the PDT was 96 hr. Similar results were obtained in experiments in which zinc sulfate was omitted from the SFRE-199-1 formulation. Therefore, it was demonstrated (Table 2) that when the SFRE-199-1 medium contained insulin and zinc sulfate, its growth supporting ability was

TABLE 3

GROWTH RATE AND CELL YIELD OF BAK CELLS IN THIRD PASSAGE IN SFRE-199-1[a]

Growth Medium	Growth Rate of Cells on Days Indicated[b]						Cell Yield per Vessel on the 7th Day Postplanting[c]
	2	3	4	5	6	7	
SFRE-199-1	+	++	+++	+++	++++	++++	2.5×10^7
HLH + 5% FBS	+	++	++	+++	++++	++++	2.4×10^7
M199 + 3% FBS	+	++	++	+++	+++	++++	2.6×10^7

[a] Cell inocula consisted of 1.5×10^5 cells/ml in 30 ml of medium per each 75-cm^2 culture flask.
[b] Method of scoring of the cell growth: ± <25% cell sheet; + = 25% cell sheet; ++ = 50% cell sheet; +++ = 75% cell sheet; ++++ = 100% confluent monolayers.
[c] Average calculated from six 75-cm^2 culture flasks.

TABLE 4

ANALYSIS OF ZN AND SE IN TC REAGENTS[a]

Type of Reagents	Detected Levels	
	Zn	Se
	μg per ml	
Fetal bovine serum	2.2	12
Horse serum	0.7	14
Protamine zinc insulin	11	20
SFRE-199-1	0.05	21
H_2O (deionized distilled)	—	—
HLH + 5% fetal bovine serum	0.2	18
EC-EDTA	—	14
HBSS	0.03	15
Se control (300 μg)	0.06	290

[a] By atomic absorption spectrophotometry techniques.

equal to the standard control cells grown in HLH + 5% FBS or M199 + 3% FBS.

Experiments were done to see whether selenium (11) would have growth promoting ability in serum-free medium. In Table 2, it is shown that 15 and 30 n*M* selenium added to SFRE-199-1 were not beneficial for cell growth but, on the contrary, demonstrated a toxic effect that reduced the cell yield and the protein content of the spent medium. An analysis of selected cell culture reagents commonly used in the laboratory (Table 4) indicated that all the reagents, with the exception of deionized distilled water, contained detectable levels of zinc and selenium.

Table 5 illustrates the effect of D-(+) galactose on the level of lactic acid synthesis in primary Bak cells grown and maintained in SFRE-199-1 and SFRE-199-2, respectively. In SFRE-199-2 maintenance medium containing 1000 mg/l of D-glucose, the level of lactic acid synthesis was 39.3% higher when compared to the level in the conventional control system. Due to this excessive lactic acid synthesis, the pH in the medium with 1000 mg/l of glucose was lower than in the conventional system. If the pH of the medium decreased to 6.8, it became difficult to maintain the cultures because of cell degeneration, thereby making the culture unsuitable for virological experiments. However, when the medium containing 1000 mg/l of D-glucose was supplemented with 1000 mg/l D-(+) galactose, lactic acid synthesis was reduced by 54.5%. In addition, the pH of the medium remained almost constant (7.3 to 7.4) during the 21-day observation period (Table 5). Upon decrease of the D-(+) galactose concentration to 500 mg/l there was a 30.0% reduction in lactic acid production; the pH maintained an average of 7.6 (Table 5), but cells did not hold up well.

Figure 1 represents the structure of Bak cells as examined by electron microscopy after growth in conventional medium or SFRE-199-1. Distinct morphological differences, apparently due to the media employed, may be seen. In cells grown on SFRE-199-1, Fig. 1*A*, the cisternae of the endoplasmic reticulum were greatly distended with an accumulation of cell products. The perinuclear cisternae were also dilated but to a lesser extent. Numerous electron-dense particles were found singly or in small clusters throughout the cytoplasm of cells grown in SFRE-199-1. They were approximately 250 to 400 Å in diameter and had

TABLE 5

EFFECT OF D-(+) GALACTOSE ON LEVEL OF LACTIC ACID SYNTHESIS IN PRIMARY BAK GROWN AND MAINTAINED IN SFRE-199-1 AND SFRE-199-2, RESPECTIVELY

Maintenance	The pH of Spent Medium on Days Indicated[a]								Lactic Acid Level[b]
	7	9	11	13	15	17	19	21	
									mg/100 ml
MEM/EBSS + 2% FBS (standard control)	7.4	7.2	7.0	6.9	7.1	7.0	6.8	6.9	129.7
SFRE-199-2 (D-glucose 1000 mg/l) without D-(+) galactose)	7.2	7.0	6.8	6.9	6.8	7.1	6.8	6.8	180.7
SFRE-199-2 (D-glucose 1000 mg/l, D-(+) galactose 1000 mg/l)	7.6	7.4	7.3	7.4	7.4	7.3	7.3	7.3	98.4
SFRE-199-2 (D-glucose 1000 mg/l, D-(+) galactose 500 mg/l)	7.6	7.6	7.5	7.6	7.6	7.6	7.7	7.6	54.2

[a] Maintenance medium was changed the 6th day postplanting when the cell sheet reached full confluency; thereafter, change of medium was carried out every 5 days. Underlined are pH values of the medium from 9–21 days in culture.

[b] Lactic acid determination was carried out on the 21-day-old cultures from 5-day-old spent medium.

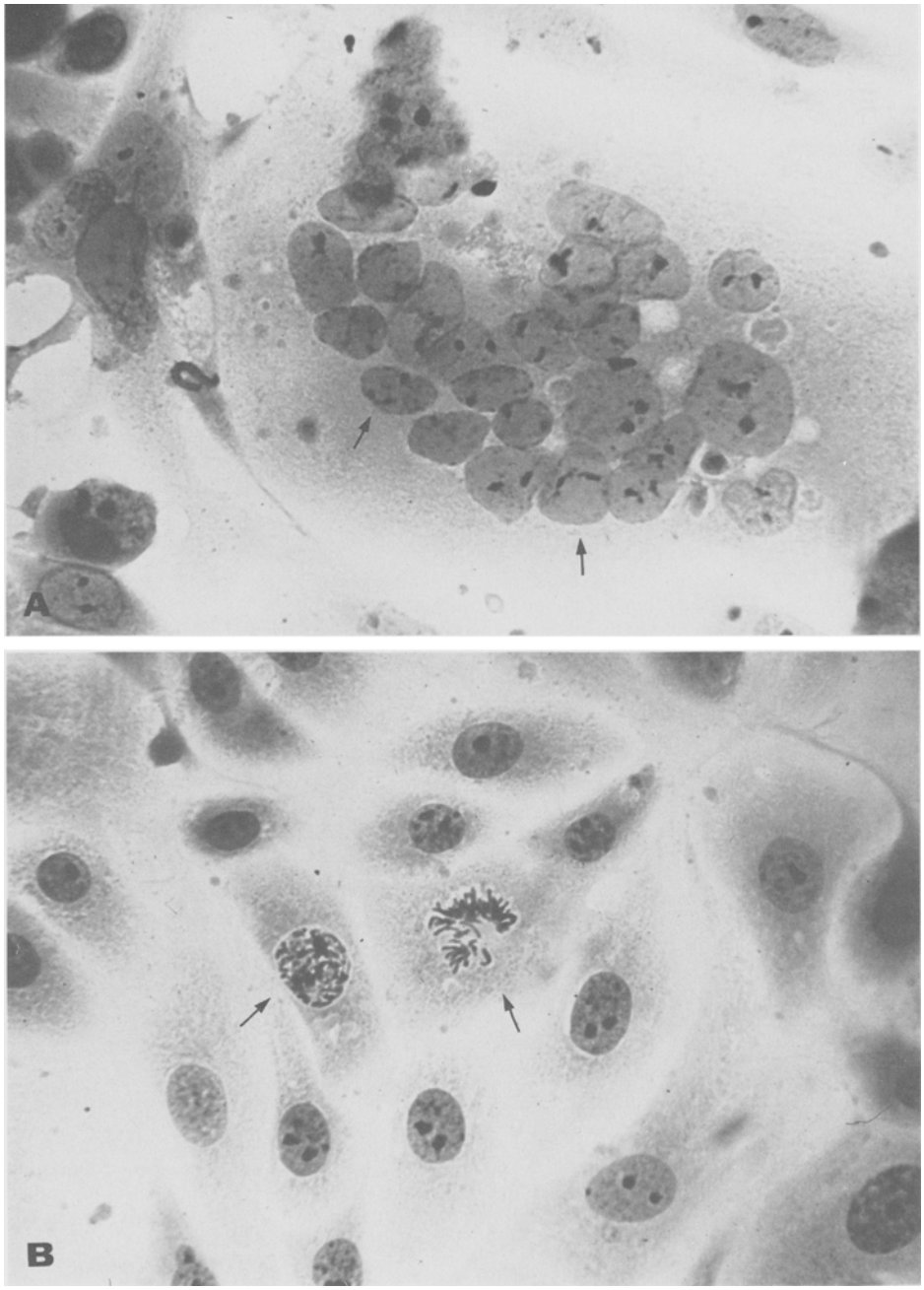

FIG. 1. Ultrastructure of Bak cells. *A*, Uninfected Bak cells grown in SFRE-199-1. Note the small, single and clustered particles (*arrows*) resembling glycogen throughout the cytoplasm. Endoplasmic reticulum (ER) and perinuclear cisternae (*arrowhead*) are distended with an accumulation of cell product. ×23,100. *B*, Uninfected Bak cells grown in the conventional system. Note the endoplasmic reticulum (*arrows*) and perinuclear cisternae (*arrowhead*) have a flattened appearance. Very few glycogenlike particles are present. ×23,100.

the appearance of glycogen. Cells grown in conventional medium also possessed similarly sized particles, but they were exceedingly fewer in number (Fig. 1*B*). The endoplasmic reticulum of these cells was flattened, as was the nuclear membrane. Other features of cells grown in chemically defined medium included a fairly well developed Golgi system, a few autophagic vacuoles, and some lipid globules.

Table 6 provides a comparison of the virus susceptibility of primary Bak cells grown in either chemically defined medium or in conventional media. Among the RNA viruses tested, echovirus type 1, coxsackievirus types B-1 and B-6, and poliovirus types 1-3 had essentially the same titers in chemically defined medium as in conventional medium. Echovirus types 2 and 3 and coxsackievirus types B-2, B-4, and B-5 had titers of 1.5 to 5.0 logs higher in chemically defined medium.

The DNA viruses tested (*Herpesvirus hominis* type 1, *H. simiae*, simian herpesvirus SA8, and simian adenovirus SV34) were not passed in Bak cells, but none showed significant differences when titrated in cells grown in the two different media.

Figure 2*A* demonstrates typical syncytium formation with intranuclear inclusions by *H. hominis* type 1 grown in Bak cells in chemically defined medium. The other viruses studied also showed typical cytopathology in chemically defined medium. Uninfected Bak cells grown in SFRE-199-1 tended to be epithelial-like in morphology (Fig. 2*B*), and typical stages of mitosis were present.

TABLE 6

VIRAL SUSCEPTIBILITY OF THE PRIMARY BAK IN SFRE-199-1 AND SFRE-199-2 SYSTEM

Viruses	Viral Titers[a]	
	SFRE-199-1 and SFRE-199-2	STD[b]
Echovirus 1	6.5[c]	6.5
2	6.0[c]	1.0
3	6.0[c]	2.5
Coxsackievirus B-1	5.7[c]	5.0
B-2	5.7[c]	2.5
B-4	6.3[c]	3.5
B-5	7.0[c]	5.5
B-6	5.5[c]	5.5
Poliovirus 1	8.0[c]	7.5
2	6.0[c]	6.5
3	7.0[c]	6.0
Herpesvirus simiae (B virus)	5.5	6.5
SA8	7.5	7.5
Herpesvirus hominis 1	3.5	4.0
Simian adenovirus SV34	5.3	6.3

[a] Titer = Log_{10} $TCID_{50}$ per 0.1 ml.
[b] STD = Conventional control.
[c] Prior titrations viruses were passaged 3 times in Bak employing SFRE-199-1 for growth and SFRE-199-2 for maintenance media.

DISCUSSION

The main purpose of this study was to ascertain the effect of eliminating the use of serum from growth and maintenance media of cultured primary baboon kidney cells and to determine the susceptibility of such cells to viruses. The use of serum-free medium would prevent the introduction of extraneous agents that may eventually find their way into the final product if present in the original serum. This would be desirable for use in virological studies and other uses in the biomedical field.

Media SFRE-199-1 and SFRE-199-2 are the results of several step-by-step modifications of M199 (8,15). Singly, these changes may result only in small improvements in cell growth. However, by combining all of the modifications used for the preparation of cell suspension from the kidneys and adding all the necessary components to the bulk formulation of the medium, it was demonstrated that primary Bak cells grew satisfactorily in a serum-free medium on both polystyrene and glass surfaces. Since the third passage Bak cells in SFRE-199-1 attained confluency with cell yields comparable to those in primary growth, this suggests that SFRE-199-1 supports the growth of primary cells beyond their survival.

To avoid uncharacterized materials as a supplement for the serum, we have concentrated on insulin as one of the main components for the serum substitute along with other nutritionally valuable components. The effect of insulin in tissue culture systems has been reported by numerous researchers, but, in most cases in which it was used as a growth supplement, it was usually in combination with uncharacterized materials (9,10,21). Other nutrients, such as zinc sulfate, sodium pyruvate, ferric nitrate, glucose, D-(+) galactose, glutamine, arginine, and some of the amino acids, were systematically adjusted to their maximum nutritional level.

Although insulin was found to be necessary in chemically defined media, its mechanism of action is not clear. Eisen et al. (37) and Parsa (38) suggested that insulin activates enzymes for glycogen synthesis during fetal development in rat liver and deposition of the glycogen in primary liver

cells. The results of our electron microscopic studies on Bak cells grown in SFRE-199-1 containing insulin indicate that they synthesize a product morphologically resembling glycogen.

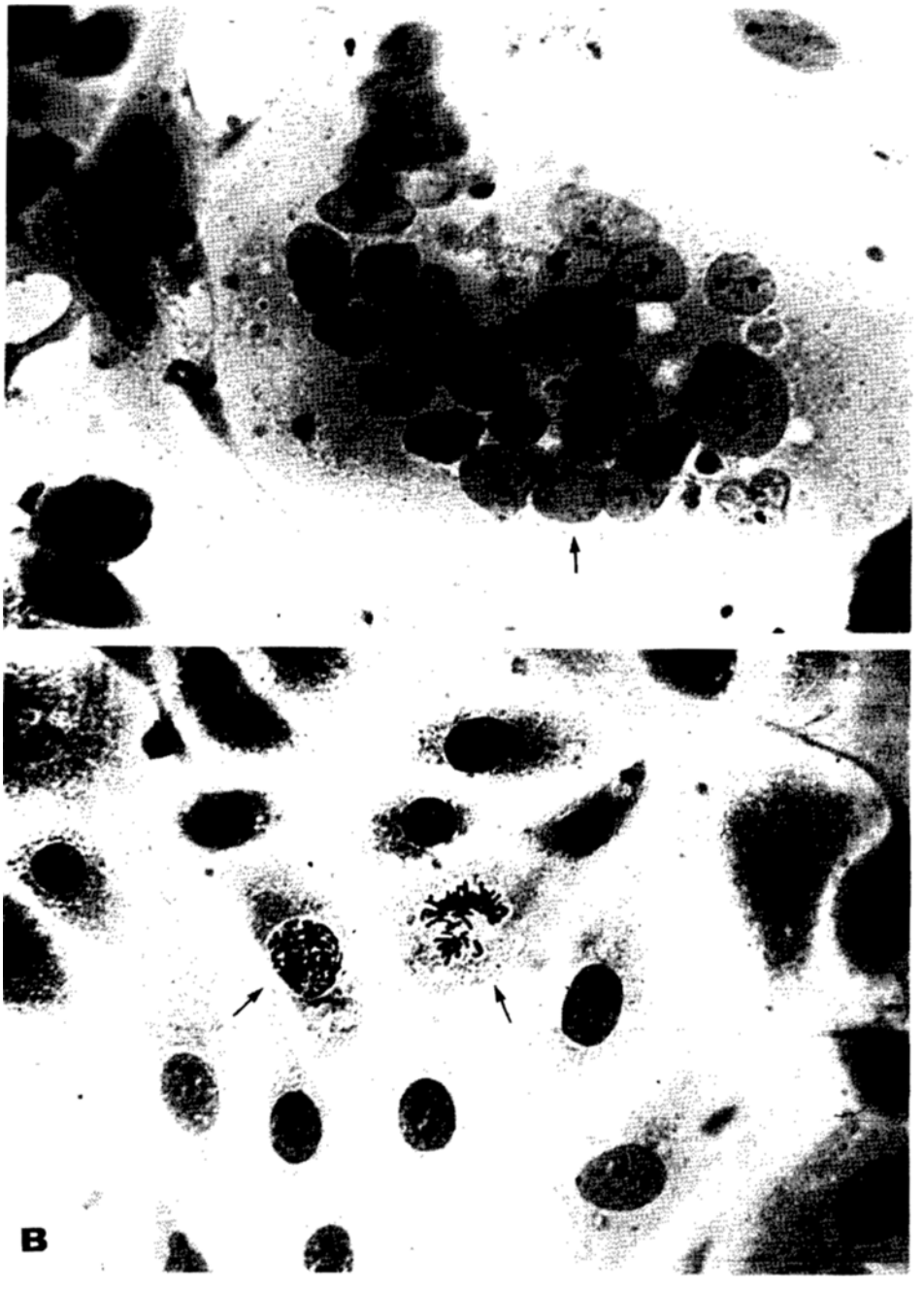

FIG. 2. *A*, Syncycial formation (*arrows*) with intranuclear inclusion bodies caused by infection of Bak cells in SFRE-199-1 and SFRE-2 system with *Herpesvirus hominis* 1. H & E stain ×242. *B*, Uninfected Bak cells grown and maintained in SFRE-199-1 and SFRE-199-2, respectively. Shown is the monolayer 8 days postplanting. Note the cells exhibiting stages of mitosis (*arrows*). H & E stain ×242.

Previously it was demonstrated that M199 fortified with insulin supported the growth of chick embryo fibroblasts, duck embryo fibroblasts, rabbit, and African green kidney cells for the cultivation and production of measles, mumps, rubella, and vesicular stomatitis viruses (22). However, in those studies the primary cell inocula suspension needed to be adjusted to 6×10^5 cells/ml to obtain full confluency, and the maintenance medium had to be changed more frequently to maintain a stable physiological pH.

Intriguing reports on the effect of selenium as an essential trace nutrient for the growth of WI-38 diploid human fibroblasts with a reduced amount of serum protein in the medium have been cited (11,29). In addition it was stated that proteins containing selenium are essential components of certain bacterial and mammalian enzyme systems (39). In our experiments, when 15 and 30 n*M* of selenium were added to SFRE-199-1, the selenium failed to improve cell growth but instead had a toxic effect on the Bak cells. Furthermore, we have found selenium in selected tissue culture reagents, with the exception of distilled water. Taking the preceding into consideration, we suggest that any amount of selenium added above the levels already detectable is not desirable in this system.

With reference to zinc, it has previously been shown (40) that serum and zinc deprivation with lowered pH caused detectable drop in DNA synthesis in chick embryo cells. Requirements for zinc and iron by cell cultures have been demonstrated previously by removing cations from medium with chelating agents and restoring growth-supporting activity with added cation mixtures (41). In our studies, zinc was found in a majority of tissue culture reagents, including fetal bovine and horse serum. It may be assumed that zinc is one of the growth-promoting elements in animal sera that also contain a great number of other uncharacterized components (2,3).

The requirements for metabolism of arginine, cysteine, glutamine, glutamic acid, glycine, histidine, and tyrosine in cell culture have been well documented by earlier investigations (16). In addition, it was demonstrated that glutamine and glycine possess an independent growth promoting effect in primary cultures of monkey kidney cells when the medium containing the above amino acids was fortified with fetal bovine serum (17).

The addition of D-(+) galactose to the SFRE-199-2 formulation resulted in a controlled pH over a prolonged period of time in culture. This is probably due to the demonstrated reduction in lactic acid production caused by the partial replacement of glucose with galactose. Other investigators have shown that prevention of partial glycolysis and excessive lactic acid accumulation enabled the prolonged maintenance of mammalian cell lines grown in a conventional system (42,43).

The higher titers obtained with enteric viruses grown in chemically defined medium compared to the titers in conventionally grown cells suggest that the titers in conventional medium could have been affected by the presence of interfering extraneous agents or antibodies in the fetal bovine serum (5-9). Alternatively, components of SFRE-199-1 and SFRE-199-2 media may have enhanced virus infectivity or stability.

In the current studies, SFRE-199-1 and SFRE-199-2 were developed specifically for the growth and maintenance of Bak cells, since it was observed that these cells were more difficult to grow and maintain than the cells from other mammalian and avian species. The benefits that could be derived from a complete chemically defined medium that would support the luxuriant growth of primary cells from different organs of a wide variety of mammals are very desirable and significant.

The results reported herein suggest that a chemically defined medium for growth and maintenance of baboon kidney is feasible. Whether or not other cells will be similarly capable of growth in such a medium needs to be determined. The obvious advantages of chemically defined medium are its inherent reduction in costs and elimination of variability and extraneous agents and their by-products found in serum-fortified media. Use of such a medium for the manufacture of various biological products is another desirable feature.

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