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The Serial Cultivation of Suspended BHK-21/13 Cells in Serum-Free Waymouth Medium[1] (39178)

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It has been established that the lipid composition of cultured cells reflects the lipid composition of their environment (1). In spite of the capacity of cultured cells to synthesize fatty acids from nonlipid precursors, fatty acids, when supplied in adequate amounts in the culture medium, are incorporated into the cells (2). Thus, in an environment free of other exogenous lipids, it is possible to quantitatively determine the fate of a given fatty acid by adding it to the culture medium.

The first step in this type of an approach is the development of a cell production system that can aid in providing sufficient quantities of cells that grow in a lipid-free environment. We have met these criteria by adapting BHK-21 cells to grow in shaker culture in the absence of serum. Previous reports of the adaptation of BHK-21 cells to serum-free media have been limited to short-term cultures (3), monolayer cultures (4, 5), or suspended cultures supplemented with complex components such as lactalbumin hydrolysate (6, 7). Our technique offers the added advantage of freezing cells for a long period of time and their recovery in the absence of serum.

Materials and methods. Cells. Baby hamster kidney (BHK-21/13) cells obtained from T. B. Stim (Yale Arbovirus Research Unit, Yale University, New Haven, Conn.) were adapted to grow as shaker cultures in Waymouth 752/1 medium (Schwarz/Mann, Orangeburg, N.Y.) as previously described (3). The code for the medium used (WO_{20}) denotes the presence of 2.5% newborn calf serum, 20 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, 20 μg sodium oleate/ml and 2 mg fatty acid-free bovine serum albumin (FAF-BSA)/ml. Portions of cultures in WO_{20} medium representing passages 142 and 165 were transferred into screw-cap Erlenmeyer flasks. Each flask received 50 ml Waymouth medium, buffered with 20 m*M* HEPES and supplemented with 2 mg FAF-BSA/ml (Pentex, Inc., Kankakee, Ill.). This medium will be referred to as W_0 medium. The inoculum for the cell cultures contained 6×10^5 cells/ml. Cellular growth was monitored by direct counts with the aid of an eosoniphile counting chamber using the dye exclusion test. Every 3 or 4 days the cells were subcultured as previously described (8). Maximum growth was obtained when the medium volume to flask ratio was maintained at 1:5. By following these procedures, a doubling time of about 70 hr was possible. Presumably, this volume relationship provides optimum exchange at the liquid–air interface.

Cell freezing. Log-phase or late log-phase cells were prepared for freezing by washing in 100 ml of Hanks' balanced salt solution. The cells were sedimented by centrifugation at 300*g* for 10 min and resuspended in Eagle's minimum essential medium containing 2× vitamins and amino acids and supplemented with 1% (w/v) FAF-BSA and 10% (v/v) dimethyl sulfoxide (DMSO). The cell suspensions were added to 1-ml cryules (Wheaton Scientific, Millville, N.J.) at a concentration of 3×10^7 cells/ml and frozen slowly at −70° in a Revco freezer.

Cells were also grown in W_0 medium supplemented with 10 μg sodium oleate/ml (WO_{10}). The conditions for growth, subculturing, and freezing were exactly as described above.

Results. The passage history of BHK-21 cells in W_0 medium and their subsequent treatment is summarized in Fig. 1. The top two horizontal rows (A and B) represent

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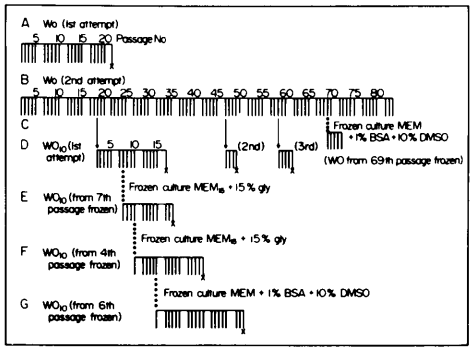

FIG. 1. The passage history of BHK-21 cells adapted to serum-free Waymouth medium supplemented with either 2 mg FAF-BSA/ml (W_0) or 2 mg FAF-BSA/ml and 10 μg sodium oleate/ml (WO_{10}). Horizontal rows are lettered A through G for the readers' reference. Each vertical line represents a passage without quantitative reference to cell growth. Occasionally serial passage was terminated (x) due to contamination or cellular toxicity. Vertical arrows (↓) indicate the passage from which subsequent passages were derived. Dotted vertical lines (⋮) indicate passages from which cells were frozen and subsequently recovered from the frozen state. The composition of the freezing solutions indicated beside the dotted lines are as follows: Eagles's minimum essential medium (MEM) with 1% (w/v) bovine serum albumin (BSA) and 10% (v/v) dimethylsulfoxide (DMSO) or Eagle's minimum essential medium with 15% (v/v) newborn calf serum (MEM_{15}) and 15% (v/v) glycerol. All cells were frozen at a concentration of 3×10^7 cells/ml and after rapid thawing, 6×10^7 cells were washed in the appropriate medium (W_0 or WO_{10}) and resuspended in 50 ml of the appropriate medium as described in the text.

two attempts to grow BHK-21 cells in W_0 medium. Row A shows subcultures of cells derived from the 142nd passage of cells adapted to grow in WO_{20} medium (8). These cultures underwent 21 passages terminating with bacterial contamination. Cells derived from the 165th passage of cultures growing in WO_{20} medium were successfully subcultured for 81 passages in W_0 medium (Row B).

The most efficient growth was obtained in Passage 32 when cells reached a density of 17.5×10^5/ml from an inoculum 6.6×10^5/ml cells after a 2-day incubation period (values not shown). Although a predictable rate of growth was never realized, total cell populations of 4.8×10^8 were frequently obtained in 400 ml culture volumes after seeding with 6×10^7 cells/ml and incubating at 36° for 4 days. The cells in these suspension cultures showed little clumping, and frequently were nonuniform in size and shape. When cells from these serum-free cultures were transferred to Falcon plastic flasks in W_0 medium, attachment as defined by Temin *et al.* (9) occurred within 1 hr, but serum (5% calf) was required to maintain cell attachment and promote cell stretching.

Row C demonstrates successful serial passages of cells obtained from the 69th passage, which was frozen in 1% FAF-BSA and 10% DMSO for 1 month at −70°. Since calf serum contains an average of 3.08 mg lipid/ml (10) and is a common supplement of cell culture media, it is possible that lipids could be a limiting factor in cell growth. We decided to investigate the effect of various amounts of oleic acid on the growth of BHK-21 cells adapted to grow in serum-free medium. Representative results are shown in Fig. 2. Cells were enumerated by direct counts at the indicated times (Fig. 2) after exposure to sodium oleate concentrations of 1, 5, 10, 20, and 50 μg/ml. Each point represents the average of duplicate counts on duplicate samples. Oleate concentrations of 1 to 10 μg/ml were stimulatory. After 190 hr incubation at 36°, cell growth

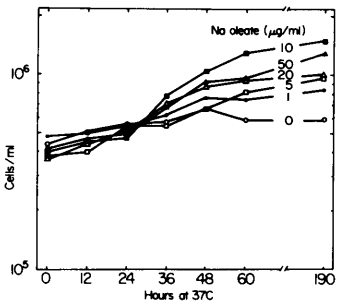

FIG. 2. The effect of various concentrations of sodium oleate on the growth of BHK-21 cells adapted to grow in serum-free Waymouth medium. Oleic acid was sterilized at 121° for 20 min in a sealed vial. After the sterile acid was converted into its sodium salt by the addition of a 10% molar excess of sterile sodium hydroxide, varying amounts of the salt were mixed with FAF-BSA and this mixture added to Waymouth medium to give the desired concentrations of oleate and 2 mg FAF-BSA/ml. Duplicate counts of viable cells were performed at the indicated times in a Speirs–Levy eosinophile counting chamber from two flasks in two sets of independent experiments.

was increased 2.5-fold in the presence of 10 μg sodium oleate/ml compared with cells growing in the absence of oleate. Higher concentrations of sodium oleate (i.e., 20 and 50 μg/ml) did not further stimulate growth.

It was of interest to determine the long range effects of BHK-21 cells growing in the presence of 10 μg sodium oleate/ml since this concentration provided maximum growth over a limited period of time. Three attempts to culture cells derived from passage numbers 18, 46, and 58 in WO_{10} medium are shown in Row D (Fig. 1). In Fig. 1, horizontal rows E, F, and G show passages of cells in WO_{10} after subjecting the parent cultures to various media used to freeze cells. The last line in Fig. 1 demonstrates that cells adapted to WO_{10} and frozen in a mixture of 1% FAF-BSA and 10% DMSO can be recovered and successfully propagated. Fifty percent of the original population were viable after thawing as judged by the trypan blue dye exclusion method. It is evident from these results that freezing in the absence of calf serum had no detrimental effect on the recovery of BHK-21 cells from the frozen state and on thier ability to be passed serially in WO_{10} medium.

Discussion. The propagation of cells in a serum-free environment has several distinct advantages over the cultivation of cells in the presence of serum: (1) There is less chance of introducing adventitious agents such as viruses and mycoplasma which are common contaminants of serum (11); (2) the cost of growing cells in the serum-free medium is less than medium with serum; and (3) studies involving endogenous lipid metabolism can be misleading when performed in the presence of serum (12). Depletion of the serum from the growth medium before chemical analysis of cellular lipids may cause stress on the normal physiological responses of the cell and in some cases lead to cell death.

Our results indicate that BHK-21 cells can be adapted to grow in serum-free medium and propagated as such for an unlimited number of passages in shaker culture. The exact function of BSA in our system has not been ascertained; however, it is believed that albumin serves as a detoxifying agent (2). Previous success by others in growing cells in shaker cultures using chemically defined media was largely attributed to the addition of methocel to the medium (14). Methocel is reputed to protect cells from the shearing forces generated by the agitation of culture fluid (15). In our cultures BSA is possibly functioning as a methocel substitute. Fatty acid-free BSA was recently analyzed by Morrison and Jenkin (16). They found that the total fatty acid content of BSA was 300 μg/g. This is equivalent to about 0.6 μg of fatty acids/ml of W_0 medium and, thus, could make a small contribution to the growth of the BHK-21 cells. In view of the reports indicating that mammalian cells can grow in lipid-free medium (17, 18), the effect (or effects) of the contaminating fatty acids in BSA on [BHK-21] cell growth is probably insignificant.

It was previously reported that when BHK-21 cells are adapted to grow in reduced amounts of serum and then transferred to Waymouth medium containing 20 μg sodium oleate/ml, rapid cell death ensues (8). However, if the cells are first adapted to grow in W_0 medium, they can subsequently be transferred directly to WO_{10} medium and serially propagated without appreciable loss of cell viability. Results from four separate experiments indicated that such transfer resulted in an average 1.8-fold stimulation in cell growth over parent cultures grown in W_0 medium (unpublished data). Upon subsequent passage in WO_{10} medium, the rate of cell growth was reduced to the same level as cells growing in W_0 medium. This phenomenon is probably a manifestation of the cellular granularity that occurs upon first exposure to oleate but which gradually disappears upon subsequent passage. Thus, by means of oleate supplementation, the fate of exogenous fatty acids in BHK-21 cells can be determined without regard to other contaminating lipid fractions.

One of the most encouraging aspects of this work was the development of a serum-free medium for freezing cells. This assured us of maintaining these cultures for long periods of time without exposure to serum.

Summary. A simple medium system was

developed to obtain growth of BHK-21 cells in shaker cultures in the absence of serum. These cells have now undergone over 80 serial passages in serum-free Waymouth medium and have been recovered from the frozen state after storage for over 1 month in medium containing 10% dimethyl sulfoxide (DMSO) and 1% bovine serum albumin (BSA).

Various amounts of exogenous lipid in the form of sodium oleate were added to cultures of cells growing in serum-free Waymouth medium. Concentrations of 10–50 μg of sodium oleate/ml had no detrimental effects on the cells as measured by trypan blue uptake. Furthermore, the cells were serially passed ten times in the presence of 10 μg sodium oleate/ml. Depletion of calf serum from the growth medium and addition of known quantities of lipids to the system provides a means of revealing subtle changes in lipid synthesis and lipid turnover during cellular growth.

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