Adaptation of BHK-21 Cells to Growth in Shaker Culture and Subsequent Challenge by Japanese Encephalitis Virus

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Baby hamster kidney (BHK-21) cells were adapted to grow in shaker culture using Waymouth medium 752/1 containing 20 mM N-2-hydroxyethyl-piperazine-N'-2'-ethanesulfonic acid buffer and supplemented with 2.5% (vol/vol) calf serum, 0.002% (wt/vol) sodium oleate, and 0.2% fatty acid-free bovine serum albumin (W02.5). Infectivity of Japanese encephalitis virus grown in the cells adapted to W02.5 approached 2 × 10^8 plaque-forming units per ml. The culture volume of infected cells was reduced fivefold 12 h after infection. This step resulted in a 10-fold increase in infectivity over that obtained from infected cultures not subjected to volume reduction.

The economical production of large amounts of cells is desirable to meet increasing demands for obtaining milligram quantities of viruses. Chemical characterization of viruses and the development of viral vaccines are activities largely responsible for this demand. Subsequent to MacPherson's and Stoker's (16) work, it was found that baby hamster kidney (BHK-21) cells were desirable for use in viral studies. Capstick et al. (2, 3) developed suspension cultures of BHK-21 cells and used them for the propagation of foot and mouth disease virus. Radlett et al. (20) greatly improved the growth conditions of BHK-21 cells in suspension culture to obtain cell densities in excess of 3.5 × 10^7/ml, thereby demonstrating the usefulness of BHK-21 cells for the commercial production of viral vaccines for animals. BHK-21 cells have proven to be susceptible to a wide range of virus groups (25), including arboviruses (24), and have a relatively stable karyotype (3, 25), and their lipids have been thoroughly characterized (8, 14, 21).

The following report describes a procedure for developing shaker cultures of BHK-21 cells in low serum concentrations and the application of this culture system for the growth of high populations of Japanese encephalitis virus (JEV).

MATERIALS AND METHODS

Cells. Baby hamster kidney (BHK-21/13) cells were obtained from T. Stim (New York State Department of Health, Albany, N.Y.) and were adapted to grow as shaker cultures in Waymouth medium fortified with 2.5% newborn calf serum (NBCS) (International Scientific Industries, Cary, Ill.).

Virus. JEV, strain M5/956 from the 10th suckling mouse brain passage, was originally obtained from Sidney Grossberg (Medical College of Wisconsin, Milwaukee, Wis.). The virus seed was prepared by infecting BHK-21 cell monolayers as described below.

Propagation of cells in suspension. BHK-21 cells were routinely cultivated in 50-ml volumes of Waymouth medium 752/1 (30) (Schwarz/Mann, Orangeburg, N.Y.) buffered with 20 mM N-2-hydroxyethyl-piperazine-N'-2'-ethanesulfonic acid (HEPES) (Sigma, St. Louis, Mo.). This medium was supplemented with 2.5% heat-inactivated NBCS, 2 mg of fatty acid-free bovine serum albumin (FAF-BSA) (Pentex, Miles Laboratories, Inc., Kankakee, Ill.) and 20 μg of oleic acid/ml (Lipids Preparation Laboratory, The Hormel Institute, Austin, Minn.) added in the form of the sodium salt (12). Hereafter, this formulation without a serum supplementation is referred to as WO and with a 2.5% serum supplementation, as WO2.5. Cell suspensions at initial concentrations of 5 × 10^6/ml were incubated at 37°C in 250-ml Erlenmeyer screw-cap flasks rotating at 125 rpm in a New Brunswick gyratory shaker. After 48 h of incubation, appropriate dilutions of cell suspensions were made and counted in duplicate, using an eosinophile counting chamber. Populations of cells routinely reached 2.6 × 10^8 cells/ml. Cells were harvested by sedimentation for 10 min at 500 × g in an International Equipment Co. (IEC) centrifuge at 22°C and transferred into fresh medium. The cells were generally diluted 1:5 for subsequent serial passage.

Virus production and quantitation. Before infection with JEV, cell suspensions were distributed into 200-ml Vitro screw-cap conical centrifuge bottles (Wheaton Laboratories, Millville, N.J.) and harvested by sedimentation for 10 min at 500 × g in an IEC centrifuge. The cell pellet was resuspended in approximately 100 ml of Hanks balanced salt solution (HBSS), washed, and resedimented as described.
above. Washed cells were infected at a multiplicity of infection (MOI) of about eight plaque-forming units (PFU) per cell and diluted to a total volume of 20 ml of Waymouth medium (752/1) per 4 x 10^6 cells. Virus adsorption was performed at 37 C for 90 min, with frequent shaking. After the adsorption period, infected cells were sedimented by centrifugation as described above, washed once or twice with 100 ml of HBSS, and resuspended to the original amount of 10^6 cells per ml in fresh WO medium or in fresh WO medium when the production of virus in serum-free medium was desired. After 36 h of incubation at 37 C, the virus suspension was frozen at -70 C for 24 h or longer, thawed, and clarified at 16,000 x g for 20 min. Portions of the supernatant fluid were distributed into smaller containers and stored at -70 C. The infectivity of the frozen virus was determined, using the plaque-overlay technique of Schultze and Schlesinger (23) with monolayers of BHK-21 cells in Linbro six-well plates (Linbro Chemical Co., Inc., New Haven, Conn.). These cells, used for the plaque assay, were obtained from 24-h-old shaker cultures. Triplicate samples (0.1 ml) of the virus, serially diluted in HBSS containing 0.2% (wt/vol) BSA, were added to the cell monolayers. After a 90-min adsorption period at 22 C on a rocker platform (Belco Glass, Vineland, N.J.), the cells were covered with 2% (15 centipoise) methyl cellulose overlay medium (23). The liquid overlay was aspirated from the cells after 72 h of incubation at 37 C. The monolayers were washed with HBSS and finally stained with 2% (wt/vol) crystal violet solution (10) containing 1% (vol/vol) formalin.

RESULTS

A schematic presentation of the development of the BHK-21 cell shaker system, from the initial attempt in MEM10 to the system as it is currently used, is shown in Fig. 1. The intent was to develop a cell culture that would grow in reduced amounts of exogenous lipids and thus ameliorate the analysis of lipids and their function during arbovirus infection. To avoid cytopathic effects on the cells due to a rapid reduction of calf serum, a method of gradual reduction of serum was used. The stepwise reduction

![Diagram](image-url)

**Fig. 1.** Adaptation of BHK-21 cells to shaker culture. MEM10, modified Eagle MEM containing 20 mM HEPES buffer, 14 mM glucose and supplemented with 10% NBCS was the primary MEM used for the cultivation of BHK-21 cells in shaker cultures. WO, serum-free Waymouth medium containing 20 mM HEPES buffer, 20 μg of sodium oleate/ml, and 2 mg of FAF-BSA/ml. MEM10 was diluted 1:2 (50% MEM10 + 50% WO) and 1:4 (25% MEM10 + 75% WO) to achieve the stepwise serum reduction. When NBCS was reduced to 2.5% or lower, MEM10 was entirely replaced by WO supplemented with the indicated amount of NBCS. The vertical arrows from the indicated passage levels represent a transfer to medium with reduced NBCS content. The number of subsequent serial passages is recorded along the horizontal lines. Horizontal arrows indicate the passage numbers of cultures that were tested for growth in serum-free WO medium which resulted in cell death (×).
of calf serum was initially accomplished by mixing modified Eagle minimum essential medium containing 20 mM HEPES buffer, 14 mM glucose (MEM\(_{10}\)) in various proportions with WO medium. Later, during the adaptation process, the addition of MEM\(_{10}\) was eliminated and replaced with WO medium containing lesser concentrations of newborn calf serum. Maximum cell growth was not achieved when the concentration of calf serum was reduced to less than 2.5% in WO medium. Each vertical line shown in Fig. 1 represents a serial passage number without reference to population density. At various steps of the adaptation process, portions of the cultures were removed (horizontal arrows) and the cells were subcultured in WO. Significant growth of the cells was not observed in WO at any step. When the calf serum concentration was reduced to 0.5%, the cells died after eight serial passages. When WO with 1% calf serum was used, the cells could be serially passed 18 times before dying. In WO containing 2.5% serum, however, cells were cultivated continuously for over 50 passages without a decrease in the cell population. Results from additional experiments revealed that concentrations of 20 mM HEPES buffer and 20 \(\mu\)g of sodium oleate/ml provided optimal cellular growth (unpublished data).

Other tests of stability of the suspended cultures were successful; i.e., cells were recovered from the frozen state (passage 18, top line, Fig. 1) and grew at comparable rates and to the same final concentration as cells that were not frozen. Removal of all traces of MEM from the growth medium (two attempts, line 5, Fig. 1) had no detrimental effect on cell density or on the growth rate.

Replication of JEV in shaker cultures of BHK-21 cells. The effect of reducing calf serum concentration on the capacity of BHK-21 cells to support the replication of JEV was examined. The virus used for inoculum was obtained by serial passage in BHK-21 cell monolayers. A five-step serial passage resulted in an increase of virus yield from 23 PFU per cell to 81 PFU per cell. Cells derived from cultures routinely grown in MEM\(_{10}\) or in WO\(_{2.5}\) were infected with this virus at the MOI shown in Fig. 2A and B. Maximum virus replication occurred at an MOI greater than 1. Virus release from both cultures began after 12 h postinfection and was virtually complete at the higher multiplicities (100, 50, and 25 PFU/cell) by 24 h postinfection. Maximum viral titers from cells grown in MEM\(_{10}\) were about 90% less than from cells grown in WO\(_{2.5}\).

Enhancement of JEV production in BHK-21 cells. The difference in virus yield from BHK-21 cells grown in MEM\(_{10}\) and WO\(_{2.5}\) was further explored. After infection of shaker cultures of BHK-21 cells by JEV, the cell growth media were replaced by MEM or Waymouth medium modified by depletion or addition of various components or supplements. The results shown in Table 1 indicate that viral yields

**Fig. 2.** Replication of various JEV inocula in BHK-21 shaker cultures adapted to either MEM\(_{10}\) or WO\(_{2.5}\). Virus samples were removed from the infected cultures at indicated times and released virus was enumerated by plaque counts.
of 568 PFU/cell were obtained from cells grown in the conventional WO₂₅ medium. Viral yields of 190 PFU/cell were increased 2.5 times to yields of 468 PFU/cell when Waymouth medium was supplemented with sodium oleate and BSA. The addition of 5% calf serum to Waymouth medium (BSA and oleate-free) reduced the virus yield. Lowest JEV yields were obtained from cells cultivated in serum-free MEM supplemented with nonessential amino acids. When MEM was supplemented with sodium oleate and BSA, virus infectivity was increased almost ninefold to 172 PFU/cell. The greatest enhancement of JEV infectivity from cells cultivated in MEM was attained after supplementation with nonessential amino acids, sodium oleate, and BSA.

**High JEV titers from BHK-21 cell shaker cultures.** The possibility of further increasing JEV titers by volume reduction of infected shaker cultures was explored. Cursory experiments in monolayers indicated that high virus titers could be obtained at 36 h postinfection when the volume of medium was reduced fivefold at 12 h postinfection and substituted with serum-free medium. These results were implemented in an experiment designed to obtain large volumes of high titered viral suspensions from BHK-21 shaker cultures. Approximately 10⁸ cells were suspended in 20 ml of unsupplemented Waymouth medium and infected with JEV at an MOI between 10 and 20. After a 90-min adsorption period at 37°C, the cells were resuspended in 1,000 ml of WO₂₅. At 12 h postinfection, infected cells were sedimented and resuspended in one-fifth the original volume with WO medium. At 36 h postinfection, infected cells were frozen at −70°C and thawed, and the virus suspension was clarified at 16,000 × g for 20 min at 4°C. The obvious effect of the volume reduction step was an increase of virus concentration from an average of 2 × 10⁶ PFU/ml to an average of 2 × 10⁸ PFU/ml (unpublished data). Further analysis of viral yields from these cultures revealed that yields had increased from an average of 188 PFU/cell in cultures not subjected to volume reduction, to 312 PFU/cell in cultures with reduced volumes. The volume reduction step, performed at an optimum time after infection, increased the efficiency of infection in cells suspended in serum-free medium.

**DISCUSSION**

There are many advantages of suspension cell cultures over monolayer cultures. First, it is not necessary to trypsinize cells in suspension culture, thereby reducing the possibility of deleterious effects on both the cells (17) and on infectious agents (1). Second, BHK-21 cells cultivated in shaker cultures do not clump readily. Sampling, therefore, becomes simplified and more reproducible. Third, less medium is used to produce the same amount of cells. For example, to produce 2.5 × 10⁶ cells in Flow 1,200-cm² roller bottles, approximately 150 ml of medium is required in two to three units. To produce the same amount of cells in 32-ounce (ca. 960 ml) prescription bottles, which yield on the average of 2 × 10⁷ cells, 13 bottles are needed requiring 455 ml of medium. In the shaker system, only 100 ml of medium in one 500-ml Erlenmeyer flask is required for the production of 2.5 × 10⁶ cells, thereby obviating manipulation and reducing the chance of microbial contamination.

The production of both group A and group B arboviruses has been attempted in BHK-21 cells (24). High yields of group A arboviruses, such as Semliki forest virus (13), Sindbis virus (4, 22, 26), and Chikungunya virus (5, 19, 31), have been reported. High yields of group B arboviruses from infected mammalian cells, however, have been more difficult to obtain. In comparison to the group A arboviruses cited above, infection of cells with group B arboviruses, such as dengue 1 (31), dengue 2, and JEV (7), and Saint Louis encephalitis (27) has resulted in 100- to 1,000-fold lower yields. Limited quantities of virus reduce the chance to perform reliable chemical characterizations.
and hamper certain biochemical studies involving the infected cell.

The high titers of JEV reported in this work result from the availability of high cell densities, volume reductions after times critical to host cell nutritional needs and before initial virus release, and the use of certain essential supplements. Three supplements were examined to determine their influence on enhancement of JEV yields: (i) calf serum, (ii) MEM nonessential amino acids, and (iii) oleate-FAF-BSA. The notion of adding or depleting calf serum in the media arose from the observation that less JEV was obtained from cells grown in MEM than from cells grown in WO₂₅, which contains 75% less calf serum. When tested with the appropriate controls, it was found that calf serum as the sole supplement at a concentration of 5% did not enhance JEV production. We have, therefore, substantiated the work of Tribble et al. (28) and Walker et al. (29) who concluded that serum is not a prerequisite for the replication of several other arboviruses in other host cell systems. The second supplement tested, a mixture of nonessential amino acids, consisting of 0.5 mM concentrations of L-proline, L-serine, L-aspartic acid, L-asparagine, L-alanine, L-glutamic acid, and glycine, neither enhanced nor inhibited JEV production in BHK-21 cells suspended in MEM with 20 mM HEPES buffer. Enhanced yields of Venezuelan equine encephalitis virus (a group A arbovirus) from suspended L-cell cultures after the addition of nonessential amino acids in serum-free medium were reported (9). The addition of nonessential amino acids in MEM, however, did not enhance JEV growth in BHK-21 cells. Our observation also substantiates the findings of Igarashi et al. (11).

The best viral yields were obtained from cells grown in WO₂₅, but oleate-FAF-BSA, supplemented in either Waymouth or MEM medium without serum, resulted in higher JEV production than from cells grown in media without these supplements. A requirement for certain lipid precursors is not surprising during arbovirus infections since the lipid composition of arboviruses (Semliki forest virus) is 30.8% by weight (15). Recently, Makino and Jenkin (18) have shown that oleic acid was a controlling factor in optimum growth of JEV in monolayers of BHK-21 cells.

The procedures used in this study provide a simpler means of growing high concentrations of JEV in BHK-21 cells. The elimination of calf serum from the medium during replication of the virus makes this system desirable for use in vaccine production and viral purification.

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