Optimization of Conditions for Production of Channel Catfish Ovary Cells and Channel Catfish Virus DNA†

TRACEY E. COLYER AND JOHN A. BOYLE*
Department of Biochemistry, Mississippi State University, Mississippi State, Mississippi 39762

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Medium supplements were examined for their effect on the growth of channel catfish ovary cells. It was found that the usual serum supplement of 10% fetal calf serum could be successfully replaced with a combination of 5% fetal calf serum and a mixture of insulin, transferrin, and selenious acid. It was also found that these cells could be grown in a more efficient manner on microcarrier beads. This type of culture produced 14 times the number of cells per milliliter of total medium used compared with the usual tissue culture flasks used for cell growth. The microcarrier system also provided for greater production efficiency of DNA from channel catfish virus, a virus that infects this cell line.

A continuous cell line, channel catfish ovary (CCO) (2), was established in 1980 for the study of channel catfish virus (CCV) (4). CCV causes a highly contagious herpesvirus infection in fingerling channel catfish (Ictalurus punctatus). The CCO cell line was established with 20% fetal calf serum (FCS) in Eagle minimum essential medium (EMEM) (2) and could be maintained in 10% FCS–EMEM (1). Growth of CCO cells was found to be most rapid at 35°C, but cells grew to a greater density at 30°C (1).

CCO cells, as well as other fish cell lines, are anchorage dependent. Microcarrier bead culture systems provide a means for growing large numbers of anchorage-dependent cells in suspension culture. Microcarrier beads can be made of polyacrylamide, glass, plastic, or cross-linked dextran. The dextran can be substituted by positively charged groups (such as DEAE) or coupled with collagen. The positively charged surface or collagen coating allows the cells to attach to the microcarrier beads and then grow. The feasibility of growing some fish cell lines on microcarriers has been studied (7).

The cost and availability of FCS hinders the use of cell lines which require it in large quantities. This becomes important when using cell lines, such as CCO, which have short population doubling times. At 30°C, CCO cell numbers double in 15.2 h, using 10% FCS–EMEM (1). To reduce costs, efforts have been made recently to define which components of serum are necessary to support growth and then add only those components.

The purpose of this study was twofold. First, we attempted to find a substitute for 10% FCS–EMEM, using new medium supplements now available. Second, we grew CCO cells on microcarrier beads. Using this system, we infected the CCO cells with CCV and purified the DNA. Yields from this system were then compared with those from plastic tissue culture flasks.

MATERIALS AND METHODS

Cells and media. CCO cells (2) were grown on 75-cm² plastic flasks (Corning) in EMEM with Hanks salts (GIBCO Laboratories) and supplemented with 10% heat-inactivated FCS (Flow Laboratories, Inc.) at 30°C.

CCO cells were seeded onto 60-mm tissue culture dishes with a 2-mm grid (Corning) at a density of 28 ± 8 cells per mm² in EMEM without serum. The medium supplements, ITS-Premix and NuSerum (Collaborative Research, Inc.), were tested in EMEM in the following combinations: 10% NuSerum, 10% NuSerum–Premix, 5% NuSerum, 2% FCS–Premix, 2% FCS–Premix–10 mM MOPS (morpholinopropanesulfonic acid), pH 7.2, 5% NuSerum–Premix, and 5% FCS–Premix. Medium on the cells was changed on day 2 or 3 as indicated. Cells were counted each day by taking pictures of three grids per dish at random and then counting the cells on the developed pictures. Pictures were taken with an Olympus BH2 microscope such that an entire grid was in the field of view.

Microcarrier culture and growth curves. Cytodex 3 (Pharmacia Fine Chemicals, Inc.) microcarrier beads were hydrated in phosphate-buffered saline, washed twice, and autoclaved. Before use, the phosphate-buffered saline was removed from the microcarrier beads and the beads were equilibrated in medium for at least 30 min. The medium was removed, and fresh medium was added to the microcarrier beads before transfer to a 250-ml hanging-bar Bellco spinner flask (model 1969).

Cells were grown in 75-cm² plastic flasks as described above and dispersed with trypsin (100 µg/ml). Cells were centrifuged for 4 min in a clinical centrifuge at approximately 1,200 × g. The pellet was suspended in 10 ml of medium; 0.1 ml was reserved, brought up to 1.0 ml, and counted in a hemacytometer. The remainder of the cells were transferred to the Bellco spinner flask. The cultures were seeded at 1 × 10⁸ to 3 × 10⁸ cells per ml with 0.5 g of microcarrier beads per 100 ml of medium.

The cells were allowed to attach to microcarrier beads in one of two ways. One was to swirl the suspension for 1 to 2 min every 30 min in approximately 30 ml of medium. Alternately, the microcarrier beads and cells were allowed to spin continuously with 40 to 50 ml of medium. The best distribution of cells was obtained with the latter method. After 4 h medium was added to 100 ml and stirred continuously at 60 rpm. Medium was replenished by letting the microcarrier beads settle and exchanging 25 to 50 ml of spent medium with an equal volume of fresh medium every day or every 2 days (as indicated).

Cell numbers were determined by removing cells from beads with collagenase (100 µg/ml). One milliliter of micro-

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* Corresponding author.
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carrier bead suspension was allowed to settle, and supernatant was removed. To the beads, 0.25 ml of collagenase was added and incubation was at 37°C for 15 min. Then 0.75 ml of medium was added and released cells were counted with a hemacytometer. Alternately, cell numbers were determined by counting released nuclei according to the method of Sanford et al. (9). Again, 1 ml of suspension was allowed to settle and supernatant was removed. To the beads, 1 ml of 1% crystal violet in 0.1 M citric acid was added and incubated at 37°C. After 1 h, released nuclei were counted with a hemacytometer. Two methods provided comparable cell counts.

**Virus infection and viral DNA purification.** One milliliter of CCV with infectivity of $10^{6.5}$, tissue culture infective doses (50% endpoint) (TCID$_{50}$) per ml was added to each 75-cm$^2$ flask. Four milliliters of CCV with infectivity of $10^{6.5}$ TCID$_{50}$/ml was added to a 100-ml microcarrier culture. The volume was reduced to 25 ml before virus was added. The virus was allowed to attach for at least 1 h. After absorption, medium was added to 80 ml and stirring was resumed at reduced speed (40 rpm). The medium used was 10% FCS–EMEM.

CCV DNA was purified from plastic tissue culture flasks by the method of Davis et al. (3). CCV DNA was purified from the microcarrier culture by a modification of this method. Medium and microcarrier beads were spun for 2 h at 20,000 $\times$ g. The supernatant was removed. To the pellet, 0.2 final volume of 1 M Tris-hydrochloride (pH 8.5)–0.5 M disodium EDTA–5% sodium dodecyl sulfate and 0.01 volume of 10 mg of protease K (Sigma Chemical Co.) per ml (in 0.2 M Tris-hydrochloride, pH 8.0, 0.1 M disodium EDTA) were added. After incubation at 60°C for 2 h, 0.002 volume of diethylpyrocarbodin (Sigma) was added, and the solution was incubated for 30 min. Then 0.2 final volume of 5 M potassium acetate was added and the solution was placed on ice for 30 min. The sample was then centrifuged at 13,000 $\times$ g for 15 min. The microcarriers were removed in this centrifugation. Two volumes of ethanol were added to the supernatant and centrifuged for 15 min at 13,000 $\times$ g. The supernatant was discarded, and the pellet was allowed to dry. The pellet was then suspended in TE buffer (10 mM Tris-hydrochloride, pH 8.0, 1 mM disodium EDTA), and 1 µg (final concentration) of RNase per ml was added and incubated for 1 h at room temperature. After RNase digestion, 2 volumes of ethanol were added and centrifuged for 10 min at 13,000 $\times$ g. The supernatant was discarded, and the pellet was allowed to dry. The DNA was then suspended in a minimal volume of TE buffer, and the concentration was determined spectrophotometrically.

Viral titers were determined by the method of Reed and Muench (8). The titer from the microcarrier culture was determined with 1 ml of the supernatant.

**RESULTS**

Different combinations of medium supplements tested fell into three groupings (Fig. 1). The first group consisted of 5% NuSerum, 10% NuSerum, or 10% NuSerum–Premix. The medium supplements in this grouping were either NuSerum exclusively or a combination of NuSerum and Premix. NuSerum does not prove to be a substitute for FCS. The completely defined conditions of medium supplements do not sustain growth of CCO cells. The second grouping of
medium supplements contained 2% FCS-Premix or 2% FCS-Premix-10 mM MOPS. This is too low a quantity of FCS even with Premix or stringent pH control to equal growth found in 10% FCS. Not shown is 5% FCS-5% NuSerum, and this combination fell into this group. The last group shows the usual growth seen in 10% FCS. The combination of 5% FCS-Premix produced a similar growth rate. Controls of 5 and 2% FCS (data not shown) were also carried out. The 2% FCS showed no growth and the 5% FCS showed poor growth similar to the second grouping of medium supplements.

Greater than 95% of cells inoculated attached to microcarrier beads. CCO cells grew on Cytodex 3, using 10% FCS-EMEM at 24 and 30°C (Fig. 2). The 5% FCS-Premix produced growth rate on the beads essentially equivalent to that with the FCS-EMEM as predicted by the preceding results. The initial growth rate in 5% FCS alone with 10 mM MOPS at 30°C was also quite good. No growth was seen in 2% FCS-10 mM MOPS.

When comparing the amount of cells produced from one plastic tissue culture flask with one 100-ml microcarrier culture when both cultures were grown in 10% FCS-EMEM, the microcarrier culture produced 100-fold more cells (Table 1). When the number of new cells produced per total volume used were compared, the microcarrier culture produced 14.3 more cells per milliliter of medium than the plastic flask did (Table 1). This volume of medium includes that changed during the growth of cells.

The microcarrier culture also allowed a large quantity of viral DNA to be efficiently purified. A 1.69-mg portion of DNA was purified from the microcarrier culture compared with 0.040 mg of DNA from the plastic flask (Table 2). This is a >40-fold increase in DNA purified with only a 6-fold increase in media used.

When the titer of CCV from a plastic flask was measured, a viral titer of 10^6.5 TCID₅₀/ml was routinely produced. Also, 0.040 mg of CCV DNA was routinely purified from one flask. The number of viral particles produced is estimated by

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**TABLE 1. CCO cell growth**

<table>
<thead>
<tr>
<th>Vessel</th>
<th>Vol of medium in vessel (ml)</th>
<th>Total vol used during cell growth (ml)</th>
<th>Cells produced</th>
<th>Cells per ml of total medium used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plastic flask (75 cm²)</td>
<td>20</td>
<td>40</td>
<td>3.9 × 10⁶</td>
<td>9.8 × 10⁴</td>
</tr>
<tr>
<td>Microcarrier culture</td>
<td>100</td>
<td>250</td>
<td>3.4 × 10⁶</td>
<td>1.4 × 10⁶</td>
</tr>
</tbody>
</table>

**TABLE 2. CCV and CCV DNA production**

<table>
<thead>
<tr>
<th>Vessel</th>
<th>Viral titer produced (TCID₅₀/ml)</th>
<th>Total vol (ml)</th>
<th>DNA purified (mg)</th>
<th>Viral particles (based on DNA produced)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plastic flask (75 cm²)</td>
<td>10⁶.5</td>
<td>4</td>
<td>0.040</td>
<td>2.8 × 10¹¹</td>
</tr>
<tr>
<td>Microcarrier (culture)</td>
<td>10⁵.5</td>
<td>80</td>
<td>1.69</td>
<td>1.2 × 10¹³</td>
</tr>
</tbody>
</table>
assuming a 100% yield of DNA. By using the molecular weight (86 × 10^6), the weight of DNA per particle can be calculated. Then the number of particles for the quantity of DNA purified is determined. Therefore, 0.04 mg of DNA represents 2.8 × 10^{11} viral particles (Table 2). Although this is an obvious underestimate of the number of viral particles produced, it may nevertheless be reasonable since the DNA purification is efficient. The infectivity ratio from a plastic flask was 1/22,000. By using the same consideration for the microcarrier culture, an infectivity ratio of 1/474,000 was obtained.

**DISCUSSION**

We have attempted to provide an easier, more efficient method for production of CCO cells and of CCV and CCV DNA. The nature of serum used in cell culture work provides inherent variation. We have reduced the serum requirement of CCO cells by half by substituting a medium supplement. This supplement, ITS-Premix, consists of 5 mg of insulin, 5 mg of transferrin, and 5 mg of selenious acid per liter of medium. In combination with 5% FCS, this supplement allowed growth rates similar to those found with 10% FCS. Further, this growth rate was sustainable after multiple passages of the CCO cells. It is used routinely in our laboratory. Use of the supplement should lower medium costs and reduce variation in growth of CCO cells.

The use of microcarrier beads provides a means for more efficient use of medium and laboratory space. The carrier culture produced 14 times the number of cells per milliliter of total media used than the usual tissue culture flasks did. It should be noted that specially designed flasks for microcarrier beads were not necessary. The simple hanging-bar suspension flasks proved adequate for these experiments. The more ambitious flask designs promise even better growth efficiencies (6). Nicholson (7) found it necessary to allow cells to attach to microcarrier beads in suspension and then transfer the beads to a flask because with some cell lines the cells would detach from the beads when grown in suspension. This phenomenon was not observed with CCV cells when Cytodex 3 beads were used. This could be because Cytodex 3 beads are collagen coated, and the cells bound more tightly to protein-coated beads than to uncoated microcarrier beads.

Cells grew very well in 5% FCS alone with 10 mM MOPS on the carrier beads. This may reflect the efficiency of growth requirement in microcarrier culture. This phenomenon has been reported for other anchorage-dependent cells growing on microcarrier beads (5). However, cells did not grow in 2% FCS–MOPS. Use of either 5% FCS–Premix or 5%FCS–MOPS could reduce total FCS needs somewhat. Whereas more rapid replenishment of media is required in each case, total FCS used to produce equivalent cell totals is only about 80% of that required by 10% FCS.

The microcarrier method also allowed a greater production of CCV DNA. One flask provided milligram quantities of DNA. The DNA was confirmed as CCV DNA by restriction digestion and subsequent agarose gel electrophoresis. No cellular DNA remained in the sample (data not shown). The viral titer from this method was anomalously low as reflected in the infectivity ratio. It seems unlikely that more defective particles would have been produced from this type of culture. Since the titer was measured from the medium but the viral DNA was extracted from the medium and cell debris, it seems more likely that large numbers of viral particles were trapped in the debris or adsorbed on the surface of the collagen-coated beads. These trapped particles can probably be liberated by sonication (7) or by treatment with a mild detergent.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**