Growth of Fish Cell Lines on Microcarriers

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Microcarrier beads were evaluated as substrates for the propagation of five anchorage-dependent fish cell lines. Growth of rainbow trout gonad (RTG-2) and Atlantic salmon cells was limited on microcarriers maintained in suspension. However, stationary microcarriers were suitable substrates for the growth of RTG-2, AS, Chinook salmon embryo (CHSE-214), and fathead minnow cells. Cell yields ranged from $2 \times 10^6$ to $2.9 \times 10^6$ cells per ml, representing 7- to 10-fold increases over the initial cell concentrations. The yield of new RTG-2 cells per unit volume of growth medium was 2.8 times greater in microcarrier cultures than in standard monolayer cultures. Northern pike cells failed to grow on microcarriers. Yields of infectious pancreatic necrosis virus propagated in microcarrier cultures of RTG-2 cells were more than twice the yields in standard monolayer cultures. The greater economy of microcarrier cultures in terms of growth vessel and medium requirements holds great promise for the large-scale production of anchorage-dependent fish cell cultures and fish viruses.

Recent increases in intensive aquaculture with the attendant disease problems as well as the realization of the importance of disease control in the restoration and management of valuable fish populations have resulted in an increased level of research on fish diseases, including viral diseases. To facilitate research on fish viruses, at least 52 different fish cell lines (K. Wolf and J. A. Mann, In Vitro 15:223, 1979) have been developed for virus isolation and propagation. The requirement for relatively large quantities of virus for research purposes as well as the likely development of vaccines for several of the more destructive viruses has created the need for mass production of fish cells. However, all fish cell lines are highly anchorage dependent and will not grow in suspension. Currently, mass production of fish cell lines is possible only with roller bottles. This method is not only laborious and expensive, but not at all satisfactory with many fish cell lines.

The recent development of microcarrier systems has provided a practical means for the mass production of higher vertebrate cells, viruses, and cell products (2). This report describes the results of our studies on the use of microcarriers for the growth of several of the most widely used fish cell lines.

MATERIALS AND METHODS

Cell cultures. The continuous rainbow trout gonad (RTG-2; 7), fathead minnow (FHM; 3), and Atlantic salmon (AS; 4) cell lines have been routinely propagated in our laboratory. The chinook salmon embryo (CHSE-214) cell line (1) was obtained from Richard McDonald, University of Calgary, Calgary, Alberta, Canada. The northern pike cell line was obtained from Olle Ljungberg, National Veterinary Institute, Stockholm, Sweden. All cell lines were propagated at 20°C in Eagle minimal essential medium with Earle balanced salts solution and supplemented with 10% heat-inactivated fetal bovine serum.

Virus. The ATCC VR299 reference strain of infectious pancreatic necrosis virus (IPNV) was propagated and quantified in RTG-2 cells as described previously (5).

Microcarrier culture. Commercially obtained diethylaminoethyl (DEAE)-dextran and polyacrylamide microcarrier beads were used. Dry microcarrier beads were hydrated in phosphate-buffered saline, washed twice, and sterilized by autoclaving. After sterilization, the microcarriers were centrifuged and resuspended in growth medium. Sterile, hydrated beads were simply centrifuged and resuspended in growth medium.

Cell stocks were produced in 150-cm² plastic flasks as described above, dispersed with trypsin, and enumerated in a hemocytometer. Microcarrier cultures were seeded at approximately $3 \times 10^7$ cells per 0.5 g (dry weight) of microcarrier beads in 100 ml of growth medium (i.e., $3 \times 10^5$ cells/ml) in a Bellco spinner flask (250 ml) on a magnetic stirrer set at 60 rpm. Some cultures were maintained in suspension continuously, and others were transferred to 150-cm² plastic cell culture flasks after allowing the cells to attach to the microcarriers (1 to 2 h). All cultures were incubated at 20°C in a humidified CO₂ incubator.

Growth medium was changed every 4 to 5 days by allowing the microcarriers to settle and exchanging 50 ml of spent medium with 50 ml of fresh medium.

Cell counts. Cells were enumerated by counting stained nuclei by the method of Sanford et al. (6).

Virus infection. RTG-2 microcarrier cultures were infected with IPNV when the cells had reached maximum density with minimum clumping of microcarrier
beads. A sample of stock IPNV was added to the microcarrier suspensions to achieve a multiplicity of infection of 0.1. Monolayer cultures of RTG-2 cells were similarly infected.

When cytopathic effect was maximum, the virus fluids were collected and clarified by centrifugation at 2,000 rpm. Cells remaining on the microcarrier beads or flasks were removed by trypsinization, sonicated, and centrifuged, and the supernatant fluid was combined with the culture fluids. Virus titers were determined as previously described (5).

RESULTS

Suspension microcarrier cultures. Microcarrier suspension cultures of RTG-2 and AS cells were prepared as described in Materials and Methods. Initial cell densities were approximately $3 \times 10^6$ per ml. Cell attachment occurred within 10 to 15 min, and cells were uniformly distributed over the surfaces of approximately 90% of the microcarriers. Invariably, cells failed to attach to about 10% of the microcarriers.

The number of RTG-2 cells increased during the first several days but leveled off and gradually declined after reaching a maximum concentration of $7.7 \times 10^6$ cells per ml at day 4 (Fig. 1). This represented less than a threefold increase over the number of initial cells. A similar culture of AS cells was even less successful. As with RTG-2 cells, growth of the AS cells occurred initially but leveled off with less than a twofold increase in numbers.

Repeated attempts to achieve significant growth of RTG-2 and AS cells on microcarriers in suspension were unsuccessful; i.e., cell densities increased initially but never exceeded two- to threefold increases over the initial concentration. Variation of cell seeding density, stirring speed, and growth medium failed to increase cell growth.

Stationary microcarrier cultures. Because the limited growth of RTG-2 and AS cells on microcarriers in suspension possibly resulted from cell damage or displacement caused by agitation, an alternate approach was attempted. The cells were allowed to absorb to the beads in 100 ml of growth medium as before, but were transferred to 150-cm² plastic cell culture flasks after 1 to 2 h. Again, the cells attached uniformly over the surface of approximately 90% of the microcarrier beads. The microcarriers in these cultures settled to the bottom of the flask in a uniformly thin layer. Representative microcarrier growth curves obtained with a variety of fish cell lines by using the modified technique are shown in Fig. 2. As in suspension cultures, there was little or no lag time in cell growth. Unlike the suspension cultures, however, four of the five cell lines studied continued to increase in cell numbers over a 1- to 2-week period, when the microcarriers were kept stationary. All cultures were discontinued before maximum cell density was reached but when the microcarriers started to clump as a result of the formation of numerous cell bridges.

The rate of growth varied with the lines, with the AS and CHSE-214 cells reaching maximum cell densities of approximately $2.9 \times 10^6$ cells per ml in 9 to 11 days. The FHM cell line reached a similar cell density, but only after 18 days. The maximum cell density of RTG-2 cells was reached in 10 days, but was lower (approximately $2.1 \times 10^6$ cells/ml) than that of the other
three cell lines. The RTG-2 cells invariably
clumped the microcarriers more readily than the
other cell lines. With RTG-2, AS, CHSE-214,
and FHM cell lines, the maximum cell densities
represented approximately 7- to 10-fold in-
creases over the initial cell concentrations. Mi-
crocarrier cultures of the northern pike cell line
were unsuccessful. The number of cells increased
only slightly above the input and then gradually
decayed.

Each of these growth studies has been re-
peated several times, using both DEAE-dextran
and polyacrylamide microcarriers. In all cases,
the results were similar to these presented here.

When cell densities reached a level where
significant clumping of the microcarriers oc-
curred, the cells were removed with trypsin,
and washed, and used to seed additional microcar-
riers in scale-up experiments.

Cell yields. To compare the efficiency of
microcarriers with that of standard monolayer
cultures, microcarriers (0.5 g per 100 ml) and
plastic flasks (150 cm²) were seeded with RTG-
2 cells and incubated at 20°C without a change
of medium. Monolayer cultures were termin-
cated and the cells were counted after 7 days, when
a dense monolayer was present. The microcarrier
cultures were terminated and the cells counted
at 11 days, when visual observation indicated
that a majority of the microcarriers were uni-
formly covered with cells. The cell yields per
unit volume are shown in Table 1. Maximum
yield obtained from standard monolayer cul-
tures was 2 × 10⁷ cells in a volume of 30 ml, or
approximately 6 × 10⁸ new cells per ml of growth
medium. The microcarrier system yielded 20 ×
10⁷ cells in a volume of 100 ml, or approximately
16.9 × 10⁸ new cells per ml of growth medium.
Thus, the microcarrier system yielded approxi-
mately 2.8 times as many new cells per ml of
growth medium as a standard culture flask.

Virus yields. To determine the efficiency of
the microcarrier system for producing virus,
standard monolayer cultures and microcarrier
cultures of RTG-2 cells were infected with IPNV
as described in Materials and Methods. When
cytopathic effect was complete, the virus was
harvested and quantified (Table 2). The yield
per cell of IPNV in the microcarrier system was
more than twice that in the standard monolayer
culture.

**DISCUSSION**

The results of this investigation demonstrate
that DEAE-dextran or polyacrylamide micro-
carrier beads are suitable substrates for the
growth of at least four (RTG-2, AS, CHSE-214,
and FHM) of the most widely used anchorage-
dependent fish cell lines. Although continued
agitation of the microcarriers in suspension cul-
tures was unsuitable for sustained growth at
least two fish cell lines (RTG-2 and AS), station-
ary microcarrier cultures yielded maximum cell
densities representing 7- to 10-fold increases over
initial cell concentrations. In all cases, except
northern pike cells, cell attachment was good;
cells remained attached throughout the growth
period; and uniform, complete monolayers were
formed on the surfaces of most microcarriers.
There was, however, some variation in cell
growth rates and maximum attainable cell den-
sities. Although no initial lag in growth was
observed with any of the four cell lines, the
growth rates of the AS and CHSE-214 cell lines
were more rapid than those of the RTG-2 and
FHM lines. The slower growth rates observed
with FHM cells probably resulted from the in-
cubation temperature of 20°C. Although FHM
cells grow relatively well at 20°C and this is the
incubation temperature frequently used, the op-
timal growth temperature for these cells is 34°C.
It was our intent to investigate the efficacy of a
microcarrier system for the culture of several
fish cell lines under uniform growth conditions.
It is likely that growth rate and cell yields can
be improved by the optimization of growth con-
ditions for individual cell lines. In all cases, a
significant amount of clumping of the microcar-

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**Table 1. Comparison of yields of RTG-2 cells
grown in standard monolayer and microcarrier
cultures**

<table>
<thead>
<tr>
<th>Vessel</th>
<th>Vol of medium (ml)</th>
<th>Cells seeded (× 10⁷)</th>
<th>Maximum yield (× 10⁸)</th>
<th>New cells produced/ml of medium (× 10⁸)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plastic flask</td>
<td>(150 cm²)</td>
<td>30</td>
<td>0.2</td>
<td>2.0</td>
</tr>
<tr>
<td>Microcarrier</td>
<td>culture (2,350 cm²)</td>
<td>100</td>
<td>3.1</td>
<td>20.0</td>
</tr>
</tbody>
</table>

**Table 2. Comparison of yields of IPNV in RTG-2
cells grown in standard monolayer and
microcarrier cultures**

<table>
<thead>
<tr>
<th>Vessel</th>
<th>Vol of medium (ml)</th>
<th>No. of cells (× 10⁷)</th>
<th>Total virus yield (TCID₅₀ × 10⁶)</th>
<th>Virus yield/cell (TCID₅₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plastic flask</td>
<td>(150 cm²)</td>
<td>30</td>
<td>1.0</td>
<td>1.6</td>
</tr>
<tr>
<td>Microcarrier</td>
<td>culture (2,350 cm²)</td>
<td>100</td>
<td>20.0</td>
<td>89.0</td>
</tr>
</tbody>
</table>

* TCID₅₀, 50% tissue culture infective dose.
riers occurred with high cell densities. Such clumping necessitated terminating cultures before the cell growth rates leveled off. Clumping was most severe with the RTG-2 cells and was the reason for the lower cell yields with this line.

The efficiency of the microcarrier system for the growth of RTG-2 cells was clearly demonstrated. There was a significant decreased requirement for growth medium in microcarrier cultures in comparison with standard monolayer cultures as evidenced by a 2.8-fold difference in the number of new cells produced per milliliter of growth medium. The microcarrier system therefore affords a greater economy not simply in reducing the requirement of numerous culture vessels, but also by reducing the amount of growth medium needed.

This study also demonstrated the efficacy of using the microcarrier system for the propagation of IPNV in RTG-2 cells. The virus yield in microcarrier culture exceeded that from a similar number of cells in standard monolayer culture. Although not compared directly with standard cultures, microcarrier cultures of AS, CHSE-214, and FHM cells also supported the replication of IPNV (data not presented).

Thus, microcarriers have been shown to be suitable substrates for the growth of a variety of anchorage-dependent fish cell lines and the propagation of virus. Advantages of this system over conventional monolayer cultures include a reduction in the cost of materials, labor, and handling, as well as better control of environmental conditions. The system also permits periodic uniform sampling for monitoring cell growth, virus production, or production of cell products. The use of microcarriers for the mass production of fish cells and viruses should have immediate benefit for research on fish viruses as well as studies of cell physiology and metabolism. The economy of mass production afforded by microcarriers also indicates their probable usefulness in fish virus vaccine production.

Additional studies should be made to determine the optimal growth conditions in microcarrier cultures for specific fish cell lines and viruses. Optimal conditions for scale-up to mass production should also be determined.

ACKNOWLEDGMENTS

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LITERATURE CITED