Production of High Efficiency of Plating Hepatitis A Virus in Primary African Green Monkey Kidney Cells (43372)

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Abstract. **High-titered hepatitis A virus, strain HM-175, was produced in primary African green monkey kidney cells (5.5 × 10^{10} tissue culture ID_{50}/850 cm^2 roller bottle). The virus preparation had an efficiency of plating of 15 particles per infectious unit. Single-cycle growth kinetics of the adapted virus indicated that after an eclipse period of 2 days, maximal yields were attained 6 days after infection.** [P.S.E.B.M. 1992, Vol 199]

Hepatitis A virus (HAV) was first adapted to cell culture by Provost and Hilleman (1). Since then, several other laboratories have reported the successful culture of HAV *in vitro* (2–5). Generally, it has been observed that with successive passages of the virus in cell culture, the eclipse period becomes progressively shortened and higher yields of virus are produced (1–4, 6–9).

The best efficiency of plating (EOP) reported was 58 particles per infectious unit (10) for a variant of HM-175 strain of HAV grown in BS-C-1 cells. Although primary African green monkey kidney (AGMK) cell cultures were found to produce good yields of HAV (3, 4, 6, 9), there has been nothing reported concerning their EOP.

In this paper, we describe the production of high yields of HM-175/HAV and their EOP in primary AGMK cells.

Materials and Methods

Virus. The cell culture-adapted HAV strain HM-175 used in this study was kindly provided by Theodore Metcalf (Baylor College of Medicine, Houston, TX) in its 22nd passage in cell culture. It had a radioimmunoassay P/N titer of 40.8 (the ratio of the radioactivity of the specimen compared with the radioactivity of negative controls), a physical particle count of 9.7 × 10^9 per ml, and, in our hands, an immunofluorescence titer of 10^5 tissue culture ID_{50} per ml.

Cell Cultures. Primary cell cultures of African green monkey (*Cercopithecus aethiops*) kidneys (Microbiological Associates, Bethesda, MD, or Flow Laboratories, Rockville, MD) containing SV (simian virus) 5 and SV 40 antisera were used. The cells were grown and maintained in minimal essential medium-L15 media consisting of Eagle's minimal essential medium supplemented with 0.736% L15 medium containing L-glutamine, 0.1 m*M* nonessential amino acids, 0.4% HEPES buffer, 0.1% $NaHCO_3$, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Fetal bovine serum was inactivated at 56°C for 30 min and added at a concentration of 10% for growth and 2% for maintenance. The cells were grown in plastic tissue culture flasks (25-cm^2 flasks and 150-cm^2 flasks) or 850-cm^2 plastic roller bottles at 37°C. Maintenance medium was usually changed at intervals of 7 days unless otherwise noted. The cells were passed by trypsinization of monolayers and plating at a density of 50,000 cells/cm^2. They were used either at first passage or at low passages (passages 2–4).

Recovery of Virus. Virus was recovered from HAV-infected AGMK cells by an adaptation of the method from Wheeler *et al.* (11). HAV-infected AGMK cells were lysed by three freeze-thaw cycles in their own culture fluids. The lysates were precipitated by adding 0.4 *M* NaCl-10% polyethylene glycol 6000 (Sigma Chemical Co., St. Louis, MO) and stirring overnight at 4°C. The precipitate was then collected by centrifugation at 10,000*g* for 30 min. The supernatant was discarded and the precipitate was suspended in 50 m*M*

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Tris buffer (pH 7.4) containing 100 m*M* NaCl (1 ml/50 ml original vol) and extracted three times with equal volumes of trichlorotrifluoroethane (Freon). The aqueous and organic phases were separated by centrifugation at 5,000 *g* for 30 min and the aqueous phases were collected and stored at −90°C.

Cesium Chloride Centrifugation. A 1.8-ml volume of 65% CsCl in TNS buffer (50 m*M* Tris buffer with 100 m*M* NaCl and 0.1% *N*-lauroyl sarcosine) was thoroughly mixed with a 3.2-ml viral sample in a 5.0-ml ultraclear centrifuge tube. This mixture, when properly mixed, gave a uniform density of 1.34 g/cm^3. The tubes were then centrifuged at 190,000 *g* in a SW50L rotor at 5°C for 24 h. Fractions were collected and assayed for HAV antigen by nitrocellulose enzyme immunoassay, and density was determined using an Abbe refractometer. Selected fractions were pooled and dialyzed against four changes of phosphate-buffered saline. The dialyzed fractions were then stored at −90°C.

Titration of Virus. Infectious virus was determined by immunofluorescence assay (12) and infectivity titers were computed by the Reed-Muench method (13).

One-Step Growth Curve. Primary AGMK cells were grown to monolayers in 25-cm^2 flasks. Virus was added to primary AGMK cells at a multiplicity of infection of 0.3–5. After adsorption for 1 hr at 37°C with agitation every 15 min, the cell monolayer was washed with phosphate-buffered saline and 5.0 ml of maintenance media (minimal essential medium-L15 with 2% fetal bovine serum) were added. The flasks were incubated at 37°C. After specific periods of incubation, infectious virus titers were determined.

Negative Staining of HAV Particles. This procedure was adapted from a method developed by Christie *et al.* (14). A length of double-sticky tape was attached to a microscope slide mounted on a large piece of filter paper with 2–3 mm of the tape sticking out on the side of the slide. The formvar-coated, carbon-stabilized, 200 mesh grids (Ted Pella, Inc., Redding, CA) were positioned along the length of the tape with the coated side up, slightly overlapping the bottom surface of each grid on the upper surface of the tape, and pressed hard enough to barely hold it in position. The sample droplet (5 μl) was applied to the grid and allowed to stand for 5 to 10 min, and removed by touching the grid rim where it was attached to the tape with a folded corner of blotting paper. The grid was immediately washed with 10 drops of distilled water from a pasteur pipette. As the washing proceeded, the blotting paper was withdrawn from the grid across the tape for a distance of 6–8 mm while maintaining contact between the grid and the blotting surface. The grid was stained by applying two drops of PTA stain (2% aqueous solution at pH 7.0 with bacitracin at 250 μg/ml) while simultaneously removing the stain with a dry corner of blotting paper. To ensure a thin and even distribution of the stain, a drop of distilled water was added and then removed with blotting paper. The grids were allowed to dry before they were removed from the tape and stored in a grid box inside a dessicator. The grids were examined with a Zeiss 10/A electron microscope.

Particle Counting. The grids were placed with the coated side inward in the 3-mm sector core chambers of a Beckman EM-90 rotor (Beckman Instruments Inc., Palo Alto, CA). Exactly 50 μl of a purified virus sample for particle counting were placed in each of the six chambers. The gasket was lightly coated with Apiezon grease (Apiezon Products Ltd, London) and positioned over the sector core. The EM-90 rotor was assembled and centrifuged in a Beckman airfuge for 30 min using an air pressure of 30 psi, which resulted in a terminal speed of approximately 95,000 rpm. After completion of the run, the grids were removed, stained with PTA, and examined. The number of particles per milliliter of the original virus suspension was calculated as follows:

$$\text{Particles per ml} = \frac{\text{particles counted (dilution)}}{\text{column height in cm (area in cm}^2\text{)}}$$

Results

Virus Passage and Growth. The data on virus passages and virus yields are summarized in Table I.

A relatively low level of infectious virus was produced during the first passage. Two additional passages at higher multiplicities of infection did not affect virus yield. However, marked increases in infectious virus production were obtained by the fourth passage through the sixth passage. Abbreviation of the incubation period from 27 days to 14 days did not result in a reduction of infectious virus yield. No gross cytopathic effect was observed in all infected cultures during the time period studied.

When the virus extracted from cell lysates was isopycnally banded in CsCl, two bands were obtained. The heavier band, with a bouyant density of 1.33 g/cm^3, was found by electron microscopy to contain only

Table I. Passage of HAV (HM-175) in AGMK Cells

Cell culture passage	Multiplicity of infection	Day of harvest	Virus yield per roller bottle ($TCID_{50}$ by IFA)[a]
1	0.0002	27	2.5×10^7
2	0.009	25	3.0×10^8
3	1	24	6.6×10^8
4	0.1	28	5.0×10^{10}
5	0.01	30	1.5×10^{10}
6	3	14	5.5×10^{10}

[a] Assayed by immunofluorescence in quadruplicates, and calculated using the Reed-Muench method. $TCID_{50}$, tissue culture ID_{50}; IFA, immunofluorescence assay.

"full" particles representing mature infectious viruses. In contrast, the lighter band (1.29 g/cm^3) was found to contain "empty" particles and represented incomplete viruses.

One-Step Growth Curve. Examination of virus growth under single-cycle growth conditions revealed an eclipse period of 2–3 days, which was followed by a logarithmic increase in virus production for 3 to 4 days (Fig. 1). Maximal virus yields are obtained at 6–8 days after infection.

Particle Counting. To determine the precision of the counting method, several counts were made on the same HAV sample. The particles were evenly distributed on the grids; no aggregates were found. The maximum deviation in six counts was 17% and the average deviation was 11% (Table II).

The counts enumerated in Table II refer to the number of virus particles found in a 1.1×10^{-7}-cm^2 area. Since the column height was 0.6 cm, the dilution was 10, and the average count was 12, the average number of virus particles per milliliter was calculated to be 1.8×10^9.

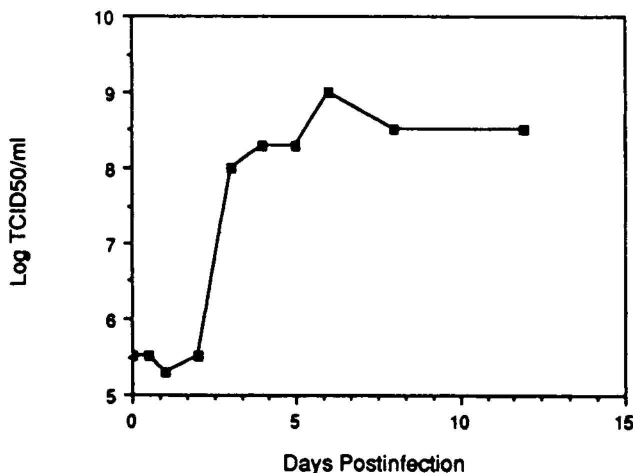

Figure 1. One-step growth curve of HM-175 in AGMK cells.

Table II. Precision of HAV Particle Counting

Replicates[a]	Particles/unit area[b]	Deviation from average[c] (%)
1	11	8
2	14	17
3	11	8
4	13	8
5	10	17
6	13	8
Average	12	11

[a] Replicate counts taken from different areas of the same grid.
[b] Number of particles counted within a 1.1×10^{-7} cm^2 area.
[c] Deviation from average computed as follows:

$$\frac{\text{Difference between actual and average count}}{\text{Average count}} \times 100\%$$

Efficiency of Plating. The virus sample counted above was previously determined to have an immunofluorescence assay titer of 1.2×10^8 infectious units/milliliter. Based on these figures, the EOP was calculated to be 15 particles/infectious unit.

Discussion

The yield of infectious virus achieved in this system after several passages in primary and secondary AGMK cells is comparable to the highest yield previously reported for HM-175 grown in AGMK cells (15).

A major characteristic in the *in vitro* propagation of HAV is the long incubation period before high yields of virus are produced. At least 3 weeks are usually required to produce maximum virus yield (4, 15). In the present studies, the single-cycle growth experiments clearly indicated that the HM-175 virus was well adapted to the cell system. The virus attained maximum titers at Day 6, and could be passed weekly without any loss of titer as long as the multiplicities of infection were high. The marked increase in infectious virus yield at Passage 4 suggested that a faster growing variant replicating more efficiently in AGMK cells was selected.

The EOP of 15 particles/infectious unit obtained here with the AGMK-adapted variant is much smaller than the 58 particles/infectious unit to date reported by Jansen *et al.* (10) for their cell culture-adapted variant of HM-175 strain HAV grown in BS-C-1 cells. This lower EOP value may be due to the production of a more efficiently replicating variant of HAV or the use of a more effective method of infectious virus recovery from cell culture.

The particle-counting method used in this study was both simple and reliable. The technique can easily be adapted for particle counting of other viruses.

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