Large Scale Production of Hepatitis A Virus in Cell Culture: Effect of Type of Infection on Virus Yield and Cell Integrity

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SUMMARY

Approaches to cell culture propagation of hepatitis A virus (HAV) have used either acute infection by passage of infected cell lysates or supernatants into uninfected cells or the passage of persistently infected cells. The findings presented here demonstrate that the growth and recovery of purified virus from foetal rhesus monkey kidney (FRhK4) cells persistently infected with HAV isolate HAS-15 decreased over a 2 to 3 month period. In contrast, high multiplicity acute infection of FRhK4 cells with purified HAS-15 HAV resulted in degeneration of the cell monolayer 2 to 3 weeks later. Large scale propagation of acutely infected cells followed by traditional picornavirus purification procedures reproducibly yielded milligram amounts of purified virus.

Conditions for the growth of hepatitis A virus (HAV) in various cell lines and under various conditions have been described in numerous reports (Provost & Hilleman, 1979; Flehmig, 1980, 1981; Flehmig et al., 1981; Daemer et al., 1981; Gauss-Müller et al., 1981; Kojima et al., 1981; Pana et al., 1982; Widell et al., 1984; Binn et al., 1984; Bradley et al., 1984; Vallbracht et al., 1984; Simmonds et al., 1985; Wheeler et al., 1986a). After initial adaptation of the virus to the appropriate cell line, the growth of HAV in cell culture has been reported to be non-cytopathic and usually results in a persistent infection. Quantification of virus present within persistently infected cells determined by cell culture infectivity or physical particle counts has varied from \(10^5\) virus particles/ml up to \(10^{10}\) virus particles/ml (Binn et al., 1984; Simmonds et al., 1985; Wheeler et al., 1986a, b). Recently, HAV in cell culture has been reported to cause degeneration of host cells (Venutti et al., 1985; Shen et al., 1986; Anderson et al., 1986; Anderson, 1987; Cromeans et al., 1987). The conditions necessary to produce the cytopathic effect in each system appear to be host cell- and growth condition-specific but the quantity of purified virus obtained under these conditions has not been reported.

Previous reports have described the adaptation and growth of the HAV isolate HAS-15 in foetal rhesus monkey kidney (FRhK4) cells (Bradley et al., 1984; Wheeler et al., 1986a, b). Large scale virus propagation methods using cells persistently infected with HAS-15 produced approximately 5 mg of purified virus from 350 l of cell supernatants and cell lysates from \(1 \times 10^6\) cm\(^2\) of cells (Wheeler et al., 1986b). However, continued propagation of these persistently infected cells in our laboratory resulted in decreased recovery of virus. This led us to investigate the various cell culture parameters required to produce reliably large (milligram) amounts of HAV.

Large scale production of HAS-15 in persistently infected FRhK4 cells was accomplished using cells split 1:3 every 4 weeks over a 3 year period. Each 6000 cm\(^2\) cell factory (Nunc) was seeded with persistently infected confluent cells from 10 T-150-cm\(^2\) tissue culture flasks
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Cells were released by trypsinization (0.05% trypsin, 0.02% EDTA for 15 min at 37 °C) and transferred into 1 l of Williams' Medium E supplemented with 2 mM L-glutamine, 0.1 mM MEM non-essential amino acids, 0.2% sodium bicarbonate, 10 mM-HEPES, 50 µg/ml gentamicin, 2 µg/ml amphotericin B and 10% heat-inactivated foetal calf serum (FCS). All components for cell culture medium were from Gibco, with the exception of FCS which was from HyClone Laboratories (Logan, Utah, U.S.A.). The supplemented medium containing the cells was transferred into a single cell factory and incubated at 37 °C. The medium was changed after 2 weeks, and cells and medium were harvested at 4 weeks. Cells were harvested by trypsinization at room temperature, fresh medium supplemented as described above was added to the cells remaining in the cell factories, and the cycle of growth, harvesting and reseeding was repeated.

Virus from large volumes of medium was concentrated as follows. (i) Polyethylene glycol (PEG) and NaCl were added to final concentrations of 6.7% and 2.3%, respectively, and after 12 to 16 h at 4 °C the precipitate was pelleted using a CF32ti continuous flow rotor (Beckman) (Wheeler et al., 1986b). The pellet was resuspended in 50 mM-Tris-HCl pH 7.5, 100 mM-NaCl (TN) and NP40 was added to a final concentration of 1%. (ii) Alternatively, the medium was concentrated 10- to 20-fold by hollow fibre ultrafiltration (100000 Mr cut-off, Amicon DC10LXR) and then precipitated with PEG and NaCl. The precipitate was pelleted at 12000 g for 30 min and resuspended in TN buffer plus NP40.

Since 80 to 90% of the virus produced in the persistently infected cells was cell-associated, cells were frozen and thawed three times in TN buffer, NP40 was added (final concentration 1%), and insoluble debris was pelleted at 12000 g for 10 min at 4 °C. The 1% NP40-soluble fraction from cells or medium was processed to further virus using a modification of the procedure originally described by Rueckert & Pallansch (1981) (Wheeler et al., 1986a). Fractions (1-0 ml) from the caesium chloride density gradient were collected from the top. Those fractions positive by enzyme immunoassay (EIA) for HAV antigen (HAV-Ag) (Wheeler et al., 1986b) and having a buoyant density of 1.30 to 1.35 g/ml were pooled, concentrated and dialysed against TN buffer. The concentrated pool was layered onto a 7.5 to 45% sucrose gradient in TN buffer and centrifuged in an SW41 rotor at 36000 r.p.m. for 90 min at 4 °C. Fractions (0.5 ml) positive for HAV antigen in the 160S region of the gradient were pooled, concentrated and dialysed against TN buffer.

Purified HAV was quantified by measuring the absorbance at 260 nm (1 mg/ml virus is 7.7 A260 units; Rueckert, 1971, 1976; Rueckert & Pallansch, 1981) while qualitative evaluation of purity was determined by polyacrylamide gel electrophoresis and negative staining electron microscopy. Purified HAV quantified by A260 evaluation (dilutions of two preparations) was used to evaluate the reproducibility and sensitivity of the HAV-Ag EIA. The results revealed that EIA-detectable HAV antigen was linear between 2 ng and 200 pg and that the lower limit of detectable HAV-Ag was 50 pg (data not shown). The radioimmunofocus assay (Lemon et al., 1983), using passage 72 to 90 FRhK4 cells and iodinated HAV antibody (HAVAB Kits; Abbott Laboratories, North Chicago, Ill., U.S.A.) revealed that approximately one-third of the physical particles were infectious and that the radioimmunofocus assay was 10^4- to 10^5-fold more sensitive than the HAV-Ag EIA for detection of virus.

Previously published recovery of purified virus from persistently infected cells was equivalent to 30 µg per cell factory (Wheeler et al., 1986b). However, subsequent purifications repeatedly yielded 10-fold less virus than expected, averaging 2 to 3 µg per cell factory. Therefore, the standardized EIA was used to evaluate various parameters to investigate the discrepancy between the reported recovery and those subsequently obtained.

The effect of FCS concentration and the time in culture on the production of virus is shown in Table 1. The production of HAV appeared to be somewhat enhanced by a reduction in FCS to 10%, but the most striking effect on virus production was due to the increase of cell culture time. Cells cultured for over 2 weeks required refeeding with fresh medium to prevent degeneration and death. This appeared to be an important co-variable that contributed to increased virus production (Table 1). Under these cell culture conditions, over 99% of the virus produced was cell-associated, and 1% was released into the cell culture supernatant (Table 1). Large scale virus
Table 1. Effect of serum concentration and growth time on HAV-Ag production on long term persistently infected FRhK4 cells

<table>
<thead>
<tr>
<th>FCS (%)</th>
<th>Time (weeks)</th>
<th>Total HAV-Ag (ng)*</th>
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<tr>
<td></td>
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<td>Cell lysate</td>
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<tr>
<td>20</td>
<td>2</td>
<td>600</td>
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<tr>
<td>20</td>
<td>2</td>
<td>726</td>
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<tr>
<td>20</td>
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<td>10</td>
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<td>636</td>
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<tr>
<td>20</td>
<td>3†</td>
<td>76</td>
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<tr>
<td>10</td>
<td>3†</td>
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<tr>
<td>20</td>
<td>4†</td>
<td>912</td>
</tr>
<tr>
<td>10</td>
<td>4†</td>
<td>1500</td>
</tr>
</tbody>
</table>

* Total HAV-Ag present in one T-150 flask of cells and medium as determined by HAV-Ag EIA.
† Medium in flasks was changed to prevent cell death.

production using 10% FCS, a 4 week growth period and refeeding after 2 weeks resulted in an increase in recovery (average) of purified HAV from 2 or 3 µg per cell factory to 10 µg.

An evaluation of HAV antigen produced over a 7 month period by persistently infected cells was performed by inoculating a single T-150 flask containing high passage FRhK4 cells (passage 258 at inoculation) with a lysate of cells infected with HAS-15 HAV (passage 23 at inoculation). Two weeks later, the medium was harvested and the cells were split 1:3. After an additional 2 weeks growth, the media from the three flasks were harvested. The cells in one flask were split 1:3; the cells from the remaining two flasks were harvested, a cell lysate was prepared by freezing, thawing and sonication, and the HAV-Ag concentrations were determined. The results suggested that the amount of virus produced during initial infection was substantially greater than that produced during the later phase of the persistent infection (Fig. 1).

Since the data in Fig. 1 suggested that initial infection of cells with HAV resulted in greater virus production, acute infection was initiated by inoculating uninfected FRhK4 cells with 300 radioimmunofocus units of purified persistently grown HAS-15 per cell. These experiments indicated that more virus was produced by the acutely infected cells and that a significant amount of virus was present in the medium, while most of virus within the long term persistently infected cells was cell-associated (Fig. 2). In addition to the increased amount of virus produced during the acute infection of FRhK4 cells, degeneration of the cell monolayer occurred approximately 2 weeks after infection (Fig. 3b), in contrast to uninfected cells (Fig. 3a) which remained intact. For comparative purposes, the long term high passage persistently infected FRhK4 cells grown under the same conditions are shown in Fig. 3(c). Inoculation of BSC-1 cells (passage 80) with 300 infectious particles per cell failed to induce any cell degeneration over a 1 month period, suggesting that this degenerative effect is virus- and cell-specific.

Large scale production of virus using acute infection was done using FRhK4 cells (passage 78 to 84) grown to confluence (using the medium described above) in 850 cm² roller bottles (Corning). Cells were inoculated with HAS-15 (160S sucrose density fraction) obtained from the persistently infected FRhK4 cells, at an m.o.i. of 300 infectious particles per cell as determined by radioimmunofocus assay. Virus was diluted in medium containing 2% FCS, added to the roller bottles and, after rotation at 37 °C for 2 h, complete medium was added with continued incubation at 37 °C. Three days later, cells from two 850 cm² roller bottles were trypsinized and transferred to a 6000 cm² Nunc cell factory as described earlier. Medium was harvested every 2 weeks, and additional uninfected FRhK4 cells from two 850 cm² roller bottles were added when cell degeneration was greater than 75%. Harvested medium was concentrated and virus purified as described above.

The quantity of purified HAV derived from FRhK4 cells acutely infected with HAS-15 averaged 50 µg per cell factory, a five- to 25-fold increase over the amount of virus obtained from the same number of persistently infected cells. In practice, there was little need to lyse cells.
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Fig. 1. Long term production of HAS-15 HAV in persistently infected FRhK4 cells. Representative samples were taken from FRhK4 cells that had been inoculated with cell culture-adapted HAS-15 and maintained as persistently infected cells (see text). HAV-Ag present in the medium and cells was quantified by HAV-Ag EIA endpoint titration. Values are the total amount of HAV-Ag (supernatant and cell lysate) at each of the designated time points.

Fig. 2. EIA endpoint titration comparison of HAV-Ag in (a) acutely infected cells (O) and medium (O) and (b) long term persistently infected cells (●) and medium (■).

because of the high degree of cell destruction that occurred over the 4 week culture period, and supernatant medium alone was used for virus purification. The purity of the recovered virus as assessed by silver staining of PAGE-separated polypeptides and electron microscope evaluation was comparable to that of the purified virus obtained from persistently infected cells.

In contrast to the extended periods needed for initial cell adaptation of virus from clinical specimens (Frösner et al., 1979; Daemer et al., 1981; Gauss-Müller et al., 1981; Binn et al., 1984; Bradley et al., 1984), cell culture-adapted virus becomes detectable by immunoassay or immunofluorescence within 1 to 17 days (Vallbracht et al., 1985; Simmonds et al., 1985; Wheeler et al., 1986a; Cromeans et al., 1987). Although persistent infection has been reported to be the most practical method to grow HAV (Vallbracht et al., 1984; Simmonds et al., 1985; Wheeler et al., 1986b), our findings indicate that over time the amount of virus produced by persistently infected FRhK4 cells decreased significantly. One explanation for this was that over a long period, the cells more sensitive to HAV growth were destroyed by infection, and a population of cells resistant to HAV infection was selected. This would explain the decreased virus production and the observation that the majority of the virus was cell-associated.

The data presented here demonstrate that growth of cell culture-adapted HAV in an acute, high multiplicity infection reproducibly yields milligram amounts of purified virus. Although a previous report from this laboratory indicated that persistent infection of FRhK4 cells can produce large quantities of virus (Wheeler et al., 1986b), the time course data and our further experience reveal that this method does not sustain high yield virus production. The continued high yield by the use of the acute infection method and the reproducible recovery of purified virus has been facilitated by the fact that HAS-15 is highly adapted to FRhK4 cells and that
Fig. 3. Degenerative effect of high multiplicity HAS-15 infection in FRhK4 cells at 2 to 3 weeks post-infection. (a) Uninfected FRhK4 cells at passage 86. (b) High multiplicity acutely HAV-infected (approx. 300 infectious particles per cell) FRhK4 cells at passage 86. (c) Long term (over 2 years) persistently infected FRhK4 cells.

These cells respond to growth stimuli such as the addition of fresh medium by increasing the number of cells available for HAV replication and growth.

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REFERENCES


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