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Persistently Infected Cultures as a Source of Hepatitis A Virus

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Primary African green monkey kidney, continuous African green monkey kidney cell line BS-C-1, and buffalo green monkey kidney cultures were infected with a uniform inoculum of hepatitis A virus (HAV). Although both the cell line BS-C-1 and primary African green monkey kidney cultures produced useful amounts of virus, HAV was detected earlier and in greater quantities in primary African green monkey kidney cultures. A persistently infected primary African green monkey kidney culture was developed. The influence of incubation time (4 to 40 days) and concentration (2 to 15%) of fetal calf serum in the maintenance medium on production of HAV by this culture was examined. An incubation period of 24 to 28 days was found to be optimal; reducing this period led to decreased yields of HAV. No significant difference in the amount of HAV produced was observed with differing concentrations of fetal calf serum. Three different methods of extraction and the effect of multiple extractions on the recovery of HAV from cell lysates were examined. Sonication was a critical factor. Two extractions yielded more than 90% recoverable virus. Yields in excess of 10^{11} physical particles of HAV per 850-cm^2 roller bottle were routine. The total yield could be increased by concentrating the HAV present in spent maintenance medium by using bentonite or organic flocculation.

Provost and Hilleman (14) first reported the successful propagation of hepatitis A virus (HAV) in *Sanguinus labiatus* liver explant and normal fetal rhesus kidney (FRhK-6) cell cultures. Virus infection in vitro results in the establishment of a noncytopathic, persistent infection (2). Virus is predominantly cell bound, with small amounts found in the culture medium. Fecal specimens from humans and feces and livers from experimentally infected animals are considered to be the best sources of virus (11). Although these sources have proven to be suitable when adequate quantities exist, clinical or infected animal samples are not always readily available in the amounts needed. A more dependable source which is capable of yielding large amounts of HAV is needed for the development of vaccines, for new and more sensitive tests to detect virus, and for biochemical and biophysical characterization of virus strains.

Propagation of HAV in a variety of cell cultures has been described previously; these cultures include FRhK-4 (5), human diploid fibroblast and human primary liver carcinoma cell (9), human amnion (13), and African green monkey kidney (AGMK) cultures (3). Binn et al. (2) recently examined the permissiveness of several cell types for isolation of HAV and the factors influencing virus growth in vitro. However, systematic studies of the development of persistently infected cultures and the conditions optimal for HAV production have not been performed. Our studies were prompted by a need to produce large quantities of HAV for research purposes.

In this study we examined persistently infected cultures as sources of virus. In this paper we describe methods developed for the routine production, recovery, and assay of relatively large amounts of HAV.

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MATERIALS AND METHODS

Virus and cell cultures. The cell culture-adapted HAV strain HM-175 which was used in this study was kindly furnished by Robert Purcell, National Institutes of Health, Bethesda, Md. This virus, which was in its 16th passage in primate cultures at the time of receipt, had an infectivity titer of $\geq 10^5$ particles per ml, as determined by an immunofluorescence (IF) assay, and a P/N ratio (counts per minute of positive sample/counts per minute of negative control) of approximately 60, as determined by a radioimmunoassay (RIA).

Primary AGMK, continuous AGMK cell line BS-C-1, and buffalo green monkey kidney (BGM) cultures were used. The primary AGMK culture was obtained as a cell suspension from M. A. Bioproducts, Walkersville, Md. The cell line BS-C-1 culture was obtained from Leonard Binn, Walter Reed Army Institute of Research, Washington, D.C. The BGM culture had been maintained in our laboratory for several years and was in its 66th passage at the time of its use.

All cultures were grown and maintained on Eagle minimal essential medium supplemented with 0.736% L15 medium containing L-glutamine, 0.1 mM nonessential amino acids, 0.4% HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, 0.1% $NaHCO_3$, 0.004% penicillin, 0.004% streptomycin, and 0.004% gentamicin. Fetal calf serum (FCS) was present at a concentration of 10% for growth and at a concentration of 2% for maintenance.

Cultures were prepared in 45-cm^2 glass bottles, 75-cm^2 flasks, or 850-cm^2 plastic roller bottles. Maintenance medium changes were usually made at intervals of 8 days for AGMK cultures propagated in glass bottles and flasks, 4 days for cell line BS-C-1 and BGM cultures propagated in glass bottles and flasks, and 12 to 18 days for AGMK cultures propagated in roller bottles. Passage of cultures was made by trypsinization of monolayers and plating of cells on a 1:2 split basis.

Recovery and concentration of virus from cell cultures. Virus was recovered from both cells and culture fluids. After removal of the culture media, 1.5-, 2.0-, and 20- to 30-ml

portions of phosphate-buffered saline (PBS) were added to glass bottles, flasks, and roller bottles, respectively. The cultures then were frozen at −15°C. During thawing, vigorous shaking or rotation of the culture vessel ensured complete removal of the cell monolayer through the shearing action imparted by the still frozen PBS. The lysates already represented a concentration of the virus originally present in the culture. The concentration of virus in culture fluids was studied by the following four methods.

(i) Method 1: MIO. Magnetic iron oxide (MIO) (15) was added to pooled media (2.5 g/liter) adjusted to pH 4 to 6. Intermittent mixing for 20 min with a glass rod at room temperature facilitated virus adsorption. The fluids were removed, and the MIO was retained at the bottom of the glass container by a magnet (electromagnetic force, 17,000 G). Virus was eluted by adding 2 to 5 ml of 2% isoelectric casein (pH 8.5), mixing for 10 min, and recovering the eluate while the MIO was retained by the magnet. The eluates were immediately adjusted to pH 7.2 to 7.5.

(ii) Method 2: organic flocculation. Culture fluids were harvested and adjusted to pH 3.5; the floc formed was pelleted by centrifugation at 10,000 × *g* for 20 min. The pelleted floc was suspended in PBS (usually 5 to 10 ml/2 liters of original medium), and the pH was adjusted to 7.2.

(iii) Method 3: organic flocculation supplemented by BE. This procedure differed from method 2 only by the addition of 0.3% beef extract (BE).

(iv) Method 4: bentonite. Concentration was achieved by adding a 0.3% bentonite suspension at a rate of 1 ml/100 ml of culture medium; the pH then was adjusted to 4.0, and the mixture was agitated periodically for 10 to 15 min. After centrifugation at 10,000 × *g* for 20 min, the supernatant was discarded, and the pellet was suspended in 6% BE at a rate of 5 ml/liter of original culture medium. Elution was carried out at pH 9.0 for 2 h at 4°C with occasional mixing. Virus was recovered in the supernatant obtained after centrifugation at 10,000 × *g* for 20 min, and the pH was adjusted to 7.2 to 7.5.

Extraction of virus from lysates of carrier cultures. Recovery of virus from cell lysates was accomplished either by chloroform extraction with Vortex mixing or by extraction at pH 9.0 with sonication or Vortex mixing. A final chloroform concentration of 10% was used, and a 2-min blending in a Vortex mixer was carried out. Sonication at 30 W for 3 min was done with a Lauda model K-2/R sonicator.

Treated lysates were centrifuged at 2,500 × *g* for 10 min; the supernatants were recovered, and the pH was adjusted to 7.2 to 7.5. Pelleted material was suspended in 10 ml of PBS, and the same treatment process was repeated. Four consecutive extraction treatments per method were performed.

RIA and reagent preparation. A solid-phase RIA was performed as described by Hollinger et al. (12), with the following modifications. Briefly, 200 ng of either chimpanzee or rabbit immunoglobulin G (IgG) anti-HAV in 100 μl of 0.1 M bicarbonate buffer (pH 9.6) was coated onto wells of polystyrene microtiter plates for 1 h at 37°C. After four washes with 2% FCS in PBS, the plates were blocked by adding 75 μl of 10% FCS in PBS per well, followed by incubation at 25°C for 30 min. Assay samples were added in 75-μl volumes and plates were incubated at 25°C overnight. After four washes with 2% FCS in PBS, 75 μl of labeled IgG anti-HAV (250,000 cpm) was added per well, and the plates were incubated at 44°C for 2 h. The plates were washed five times with 0.01% Tween 20 in PBS, covered, cut, and counted with a gamma counter.

Antibody to HAV obtained from sera from an infected chimpanzee and from a human with hepatitis was generously supplied by Howard Fields, Centers for Disease Control, Atlanta, Ga. Rabbit hyperimmune serum was obtained after four biweekly intramuscular injections of an immunogen containing 10^{10} purified HAV particles in Freund complete adjuvant. Serum was obtained 14 to 35 days postimmunization.

Chimpanzee and human sera were fractionated with 18% sodium sulfate and suspended in PBS, and an additional 12% sodium sulfate fractionation step was performed. Human and chimpanzee IgG was purified by ion-exchange chromatography, using DEAE-cellulose as previously described (4). Flowthrough fractions giving absorbance at 280 nm were pooled and concentrated by ultrafiltration. The degree of concentration achieved was determined spectrophotometrically by using an extinction coefficient of 13.5 for a 1% solution at 280 nm.

Rabbit IgG was purified by protein A-Sepharose CL4B chromatography. Antibody was eluted with 0.1 M acetic acid adjusted to pH 3.4. Protein-containing fractions (monitored by absorbance) were pooled and neutralized immediately with 1 N NaOH. After overnight dialysis against PBS at 4°C, antibody was concentrated by ultrafiltration. Insoluble and aggregated material was removed by centrifugation at 2,000 × *g* for 10 min, and antibody concentration was determined as described above for chimpanzee and human antibody.

A 10-μg portion of purified human IgG anti-HAV was labeled with 10 μCi of $Na^{125}I$ by the chloramine T method (10). Non-protein-associated ^{125}I was separated on PD-10 columns. More than 95% of the ^{125}I-labeled IgG anti-HAV was precipitated with trichloroacetic acid at a final concentration of 10%. Labeled IgG for use in the RIA was diluted with a diluent composed of 50% PBS, 40% FCS, and 10% normal human serum.

RIA endpoint titration. Assay endpoints were assumed to be directly proportional to the number of physical particles of HAV present in a sample. Previous studies showed that RIAs were positive if P/N values of ≥2.1 were obtained. We reasoned that tests performed on a series of dilutions of a sample would result in declining P/N values and that quantitation of HAV numbers present could be obtained by using a P/N value of 2.1 as a titration endpoint.

The number of physical particles per unit volume required for a positive test (P/N ratio of ≥2.1) was determined by constructing a curve showing the relationship between the number of physical particles present and the P/N ratio determined. To produce this curve, twofold dilutions of a purified HAV preparation containing a known number of physical particles per unit volume were prepared, and RIAs for each dilution were performed in triplicate. The resulting curve is shown in Fig. 1. The endpoint served as a standard frame of reference from which the dilution factor, and thus the number of physical particles present, could be calculated.

The minimum number of physical particles detectable in a positive RIA was determined to be 8×10^6 particles per ml. A value of 2×10^7 HAV particles per ml was chosen as the standard titration endpoint. In each assay performed, standard known particle preparations containing 2×10^7 and 1×10^7 particles per ml were included as controls; the former always gave P/N ratios of >2.1.

HAV physical particle counts. Particle counts were made by using a calibrated RCA model EMU 3F electron microscope. Samples (20 to 50 μl) were dried onto agar disks (2%

Difco special Noble agar in 0.85% saline). Parlodion (0.75% in amyl acetate) was added, spread, drained dry, and stained with 1% ammonium molybdate. The film was transferred to a grid, and a particle count was obtained as described by Smith and Melnick (16).

IF. Microscope slide cultures to be examined for the presence of HAV were washed twice with Tris-saline buffer and allowed to air dry. The cultures were fixed with anhydrous acetone for 10 min at −20°C. Fixed cultures were washed twice with Tris-saline buffer, and 25 μl of a 1:10 dilution of human anti-HAV was added. The cultures were incubated for 40 min at 37°C in a moisture chamber and washed twice with Tris-saline buffer, and 25 μl of fluorescein isothiocyanate-goat anti-human IgG conjugate was added. After 40 to 60 min of incubation at 37°C, stained cultures were washed twice with Tris-saline buffer, rinsed with distilled water, and covered with Elvanol-PBS. The slides were examined with a Zeiss UV microscope.

The controls used for the IF test included infected cultures stained only with fluorescein isothiocyanate-conjugated goat anti-human IgG, uninfected cultures stained exactly as the infected cultures were, infected cultures stained only with anti-HAV, and infected cultures stained with normal human IgG and fluorescein isothiocyanate-conjugated goat anti-human IgG.

RESULTS

Establishment of a persistently infected culture. A suspension of AGMK cells with simian virus 5 and simian virus 40 antisera was used to prepare primary cultures in 75-cm^2 flasks; 1 ml of a 1:10 dilution of HAV strain HM-175 was added to several 12-day-old monolayers and allowed to adsorb for 2 h at 37°C. The virus preparation was sonicated at 100 W for 1 min; this was followed by filtration through a serum-treated membrane filter prior to use. Maintenance medium was added without previous washing of the monolayers, and flasks were incubated at 36°C. The maintenance medium was changed 9 days later (21-day-old cultures), and cells from some of the cultures were harvested 10 days after the medium change (31-day-old cultures). A cell lysate was prepared by three freeze-thaw cycles and stored at −20°C. Subsequent passages of other cultures were made at intervals varying from 5 to 9 days. The development of a persistently infected culture and the effect of weekly passage upon virus production are shown in Table 1.

TABLE 1. Development of a persistently infected AGMK culture and HAV production[a]

Cell culture passage	Growth in cell culture (days)	Virus yield (P/N ratio)
4	17	22.8
5	8	14.6
6	9	30.7
8	6	27.7
9	6	26.7
10	9	37.8
11	7	13.4
12	7	6.7
13	5	3.6

[a] Cultures were passaged without further inoculation of HAV after the initial infection.

RIAs showed that the AGMK culture was infected by the initial addition of virus and that subsequent passage of the culture without further addition of virus resulted in regular production of virus. P/N values of undiluted lysates indicated good virus production for culture passages 4 through 10. Culture passages 11 through 13 were accompanied by a continuing decline in numbers of virus produced; virus production fell rapidly in each of these passages. We found that passage of a culture at weekly intervals rapidly depleted the virus numbers detectable in RIAs. This depletion could be reversed, and the virus numbers produced could be increased, by allowing 24 to 30 days of culture maintenance between passages.

Production of HAV in persistently infected AGMK cultures. The production of virus in persistently infected AGMK cultures was studied to determine the influence of prolongation of culture maintenance upon the amount of virus produced. A total of 30 75-cm^2 flasks were seeded with persistently infected cells, and monolayer formation was obtained. Confluent monolayers formed by the second day of incubation. Three flasks were removed every 4 days and were tested for virus over a 40-day interval. The cells were harvested, lysates were prepared, and RIA endpoint titrations were made (Fig. 2).

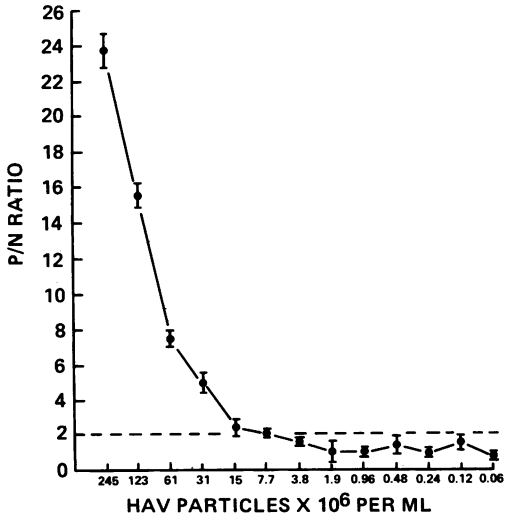

FIG. 1. Determination of the RIA endpoint value. The bars represent 1 standard deviation (n − 1).

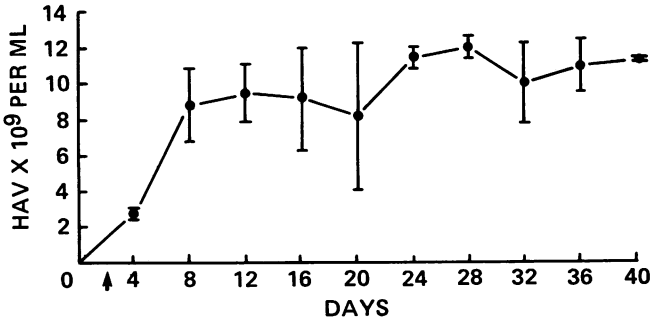

FIG. 2. Production of HAV by persistently infected AGMK cells. Monolayer formation was complete within 48 h (arrow). The bars represent 1 standard deviation (n − 1).

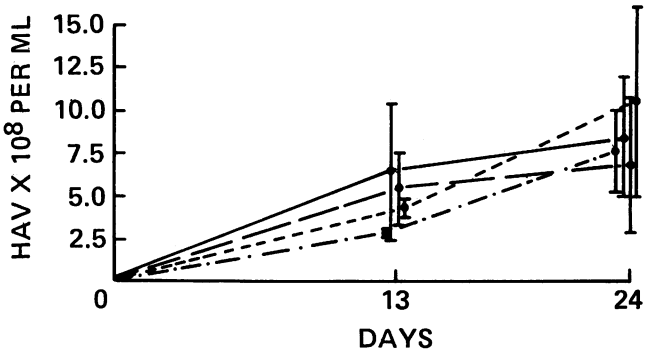

FIG. 3. Effect of FCS concentration on the production of HAV in persistently infected AGMK cells. FCS was tested at concentrations of 2% (vol/vol) (dashed and dotted line), 5% (vol/vol) (solid line), 10% (vol/vol) (long dashed line), and 15% (vol/vol) (short dashed line). The bars represent 1 standard deviation ($n - 1$).

It was readily apparent that virus production could be optimized by prolonging culture time. Maintenance of a culture for 24 to 27 days resulted in peak virus production. Prolongation of a culture beyond this time was nonproductive. As indicated in both Table 1 and Fig. 2, cells harvested after 5 to 7 days of incubation resulted in poor virus yield and, if carried out sequentially, ultimately led to a decline in virus production.

Virus production was followed by IF tests as well as RIAs; control and infected cultures were examined at 5, 7, 9, 12, and 14 days postinfection. Minimal (plus/minus) fluorescence was usually visualized after 5 days, and definite positive results were detectable after 7 days with infected cultures. Fluorescence was intracytoplasmic only and exhibited a discrete granular appearance. The number of fluorescing foci increased with time, with an estimated 80% of a culture positive by day 14. Control cultures remained negative throughout the 14-day test period.

The influence of nutritive quality of a medium upon virus production was studied by supplementing culture maintenance medium with increasing amounts of FCS. A total of 28 45-cm^2 bottles were seeded with persistently infected AGMK cells. After monolayers were confluent, the maintenance medium was supplemented with 2, 5, 10, or 15% FCS. Three and four bottles for each serum supplementation were harvested at days 13 and 24, respectively. Cell lysates were prepared, and RIA endpoint titrations were performed (Fig. 3).

There was little indication that virus production in persistently infected AGMK cultures was influenced by the amount of serum in the maintenance medium. The relatively small differences in the numbers of physical particles produced by the four sets of serum-supplemented media became insignificant when the degree of variation between multiple particle counts indicated by standard deviation bars was taken into consideration.

Production of virus in BGM cultures was investigated by using an 81st passage culture to prepare monolayers in six 75-cm^2 flasks. A 1:10 dilution of an HAV suspension with an RIA value of 16,685 cpm/75 μl was made, and 0.3 ml was adsorbed to the monolayer for 3 h at 36°C. Maintenance medium supplemented with 2% FCS was added to test and control plates alike, and the cultures were incubated at 36°C. One test flask and one control flask were harvested for cells at 14, 20, and 28 days, and RIAs were performed on cells and culture supernatants (Table 2).

Virus production was found to have occurred by 14 days and did not increase notably thereafter. Virus was essentially cell associated, with small amounts appearing in culture supernatants. Virus from an original BGM culture infection yielding an RIA value of 3,787 cpm/75 μl (P/N value, 33) was diluted 1:3 and used to infect other BGM cultures. These cultures were maintained for 14 days before harvesting; lysates were prepared and, when tested, yielded 918 cpm/75 μl (P/N value, 8.0).

Production of virus in BGM cultures was compared with virus production in fresh AGMK and cell line BS-C-1 cultures. Each of these cultures was grown in 45-cm^2 bottles and inoculated with 1 ml of a 10^{-2} dilution of seed virus (1,342 cpm/75 μl), followed by a 2-h adsorption at 37°C. One bottle of AGMK cells and three bottles each of BS-C-1 and BGM cells were examined for virus at 4, 8, 12, 16, 20, and 24 days postinfection (Fig. 4).

Virus production was detected in cell lysate samples from AGMK and cell line BS-C-1 cultures by day 8, and the amount of virus produced in these cultures increased thereafter. No virus production was detected in BGM lysates at any time. Subsequent studies based on the positive results shown in Table 2 indicated that productive infection of BGM cultures required larger numbers of virus than the numbers which were present in the comparative study shown in Fig. 4. The comparative study showed that AGMK cultures produced more than twice as much virus as cell line BS-C-1 cultures and was the culture of choice for the production of HAV.

Extraction of virus from cell lysates. Chloroform extraction was compared with extraction carried out at pH 9.0, with and without sonication. AGMK cells from nine 850-cm^2

TABLE 2. HAV production in BGM cultures

Days postinfection	P/N ratio in infected cultures		P/N ratio in noninfected cultures	
	Cells	Supernatant	Cells	Supernatant
14	86.8[a]	12.1	2.0	0.4
20	95.6	10.6	0.9	0.5
28	90.2	10.5	0.3	0.3

[a] P/N ratio of sample against PBS control.

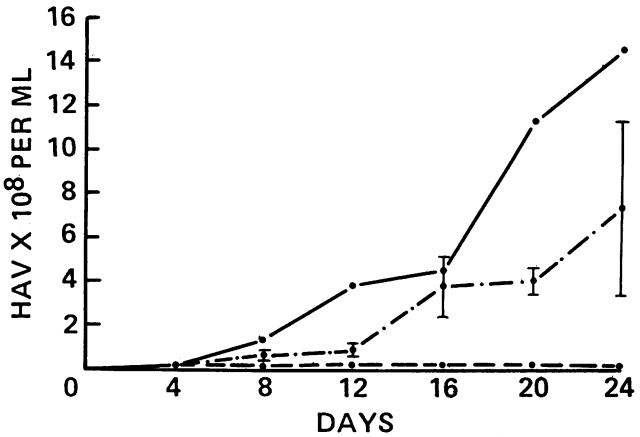

FIG. 4. Comparative yields of HAV from AGMK (solid line), BS-C-1 (dashed and dotted line), and BGM (dashed line) cell cultures. The bars represent 1 standard deviation ($n - 1$).

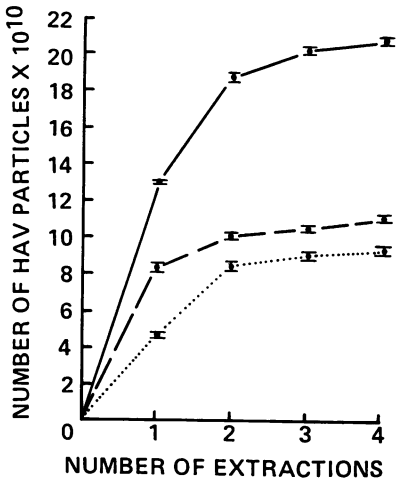

FIG. 5. Extraction of HAV from cell lysates by pH treatment and sonication (solid line), pH treatment and blending in a Vortex mixer (dashed line), and chloroform treatment and blending in a Vortex mixer (dotted line). The bars represent 1 standard deviation. Where the error was too small to represent graphically, it has been omitted.

roller bottle cultures maintained for 24 days were harvested and pooled, and PBS lysates were prepared. Three 30-ml volumes of lysate per extraction method were used, and the extracts were evaluated by RIA endpoint titration (Fig. 5).

Extraction either at pH 9.0 or after addition of chloroform resulted in recovery of about 10^{11} physical particles. However, a twofold increase in recovery effectiveness was obtained with sonic extracts at pH 9.0, making this the method of choice when virus yield alone was considered. Chloroform extractions removed some of the cellular debris found with preparations extracted at pH 9.0 and gave noticeably cleaner virus suspensions. Since each curve in effect represents average cumulative amounts of HAV extracted from an equivalent of one 850-cm^2 roller bottle by each of the three methods, the data indicate that at least 10^{11} physical particles are obtainable from the cell lysates of one 850-cm^2 roller bottle.

A comparison of the levels of virus recovery in each of the four consecutive extractions showed that most of the virus present in a 30-ml lysate appeared in the first two extractions, regardless of the method used. Based on these data, two extractions were considered sufficient for recovery of most of the virus present.

Concentration of non-cell-bound virus in culture media. Although HAV produced in persistently infected AGMK cultures remained predominantly cell associated, some virus was found in the culture fluid. Since the combined volume of media recovered at one time from several roller bottles used for virus propagation was more than 2 liters, considerable virus could be recovered if a suitable method of concentration could be found. Bentonite, organic flocculation with and without BE supplementation, and MIO procedures were examined for their virus-concentrating effectiveness (Table 3).

Flocculation methods were more effective than the MIO procedure. Bentonite and organic flocculation, especially when supplemented with BE, appeared to be useful. Our results showed that additional virus could be recovered from cast-off culture media and emphasized the advantage to be gained by taking the time to carry out these relatively simple procedures.

DISCUSSION

The use of a persistently infected AGMK culture was found to be advantageous for large-scale production of HAV. Development of a persistently infected culture was easily accomplished, and its maintenance was routine. The ease with which a persistently infected culture was obtained undoubtedly was due to the use of strain HM-175, which previously had been adapted to growth in primate cultures (3). The persistently infected culture has been in continuous use in our laboratory for the past 2 years. It continues to yield high titers of HAV. Little difference between virus yields from passages 2 through 33 has been detected.

Use of the RIA endpoint titration was crucial to our studies of virus production. We needed to be able to differentiate between differing amounts of virus, and our regular solid-phase RIA was inadequate for this purpose, particularly when samples contained high concentrations of

TABLE 3. Comparison of four methods for concentration of the virus present in culture media

Concn method[a]	Eluent	Final sample vol (ml)	Concn factor	Final sample titer (P/N ratio)
Trial 1 (initial pool, 1,600 ml)				1.9
Bentonite	6% BE, pH 9.0	5.0	80	16.8
Organic flocculation	0.5 M glycine, pH 9.0	5.0	80	7.3
Organic flocculation + 0.3% BE	0.5 M glycine, pH 9.0	5.0	80	9.2
MIO	3% BE, 0.5 M glycine, pH 9.0	2.8	143	4.1
Trial 2 (initial pool, 3,200 ml)				1.8
Bentonite	3% BE, 0.5 M glycine, pH 9.0	3.0	133	34.3
		3.0	133	34.0
Organic flocculation	3% BE, 0.5 M glycine, pH 9.0	3.0	133	30.0
		3.0	133	28.0
Organic flocculation + 0.3% BE	3% BE, 0.5 M glycine, pH 9.0	3.0	133	36.0
		3.0	133	31.2
MIO	3% BE, 0.5 M glycine, pH 9.0	2.8	143	4.8
		2.8	143	3.1

[a] One examination per method was made in trial 1, and duplicate examinations per method were made in trial 2. In each test we used a 400-ml volume of the initial pool.

HAV or cell debris. Anders et al. (1) also commented on the need for endpoint titrations when quantitative determinations of cell-bound HAV are desired. Both endpoint titration methods represent useful quantitative procedures. Unlike the method used by Anders et al. (1), however, our method allowed quantitation of virus as numbers of physical particles per milliliter.

We learned early not to hurry virus production. Initial weekly passage of persistently infected cultures eventually resulted in drastically lowered virus yields. Prolongation of culture maintenance to 24 to 28 days between passages resulted in increased virus production. Virus production declines (obtained with 14-day cultures, for example) could be reversed by increasing culture maintenance to 28 days or longer.

Infection of three primate cultures (AGMK, cell line BS-C-1, and BGM cultures) under identical conditions resulted in distinct differences in the amount of virus produced, as well as in the rapidity of production. Although AGMK and BS-C-1 cultures produced useful quantities of virus, the former produced much more virus and virus accumulation was more rapid, an observation also made by Binn et al. (2). Infected cell IF was first observed at day 5 with AGMK cultures, whereas BS-C-1 cells were still negative at day 12 in the studies of Binn et al. Our detection of plus/minus fluorescence after 5 days and positive fluorescence after 7 days in persistently infected AGMK cultures was virtually identical to their results.

The rapidity of HAV growth and the detectability upon passage of persistently infected AGMK cultures were not influenced as markedly as previously reported for passage of HAV of human origin in human embryonic kidney or human embryonic fibroblast cultures (7). The differences between the results of our study and the study of Flehmig et al. (7) were attributed to the different strains of HAV used. The strain HM-175 virus used in our studies had been adapted to growth in primate cells, whereas Flehmig et al. (7) worked with nonadapted virus isolated directly from human stools. An eventual 4- to 5-day minimum period needed for detection after adaptation of the virus to the cell culture system in use was reported in both studies.

The BGM culture that was nonproductive in the comparative growth experiment was later shown to be capable of infection and production of virus if a sufficiently large infecting dose was used. Passage of an infected BGM culture resulted in production of virus in 2nd passage cells, but at a reduced level. Gauss-Muller and Deinhardt (8) and Binn et al. (2) reported a decrease in detectable amounts of HAV with increasing levels of FCS. No significant difference in virus production by cultures maintained on media supplemented with 2, 5, 10, and 15% FCS was noted in our studies. A comparison of these results is difficult in view of differences in both experimental design and assay quantitation.

Our routine production of cell-bound virus per 850-cm^2 roller bottle in excess of 10^{11} physical particles of HAV is higher than the estimates made by workers in other laboratories for strain HM-175 production in AGMK cells (2) or strain H-141 in FRhK-4 cells (1). Our finding of virus in culture fluids at levels that were 1 to 10% of the quantities produced in cells increased the total virus production recoverable. The levels of virus recovery in culture fluids resembled the values reported by Daemer et al. (3) for the growth of strain HM-175 in AGMK cells. However, it should be noted that both Binn et al. (2) and Anders et al. (1) found approximately equal titers of virus in cell and medium supernatant fractions using strain HM-175 in BS-C-1 cells and strain H-141 in FRhK-4 cells, respectively. Also, Flehmig et al. (6, 7) reported a greatly increased release of virus into culture fluid upon adaptation of HAV to human embryonic kidney cells.

The virus yields in our studies were greater in part because special attention was directed toward optimization of cell lysate extraction and culture fluid concentration methods. The key differences in our studies were sonication at pH 9.0 with multiple extractions for cell lysates and either organic flocculation or bentonite-mediated virus concentration from culture fluids.

The advantages of using persistently infected AGMK cultures for large-scale production of HAV compared with the practice of infecting fresh cultures can be summarized as follows: (i) persistently infected cultures produce greater quantities of HAV; (ii) accumulation of intracellular virus is more rapid; (iii) serial passage of persistently infected cultures is less time-consuming; and (iv) adventitious viruses are not a problem in established cultures.

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