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Primary Isolation and Serial Passage of Hepatitis A Virus Strains in Primate Cell Cultures

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Although several primate cell types have been reported to support replication of hepatitis A virus, optimal conditions for the isolation and production of quantities of virus have not been defined. We therefore examined seven different primate cell types for their ability to support replication of primate-passaged and wild-type virus as reflected by intracytoplasmic accumulation of viral antigen (direct immunofluorescence and radioimmunoassay) and propagation of cell culture-adapted virus. Of the cells tested, low-passage African green monkey kidney (AGMK) cells were most sensitive for initial isolation. Viral replication was documented after inoculation of AGMK cells with seven of nine hepatitis A virus antigen-positive fecal specimens (from seven epidemiologically distinct sources). With six inocula, virus was successfully passed in serial cultures. AGMK-adapted virus was readily propagated in continuous AGMK (BS-C-1) cells. The optimal temperature for the growth of virus in BS-C-1 cells was 35°C. Viral release into supernatant fluids was documented in the absence of any cytopathic effect, and infectivity titers in supernatant fluids 21 days after inoculation (50% tissue culture infective does [$TCID_{50}$], $10^{6.0}$/ml) equalled or exceeded those in the cell fraction ($TCID_{50}$, $10^{5.5}$/ml). Cells maintained in serum-free media readily supported viral growth, with yields of virus ($TCID_{50}$, $10^{6.5}$/ml) equal to or greater than those obtained with cells maintained in 2% fetal bovine serum.

The successful isolation and propagation of hepatitis A virus (HAV) in cell cultures was first reported by Provost and Hilleman in 1979 (17). As the virus was not cytopathic, their success was attributed in part to the development of sensitive immunological tests for HAV and to the use of readily cultivated, marmoset-passaged virus. However, the primary isolation of HAV from specimens of human origin remains a difficult, prolonged, and uncertain procedure (3, 5–7, 10, 16, 17). Although a variety of primate cell types have been utilized for primary isolation and propagation of the virus, there have been few systematic attempts to compare the degree of permissiveness of various available cell types. In addition, there are few reports describing the effect of temperature, media composition, or serum concentration on the yield of cell culture-adapted strains of virus. We report here our observations on the range of cell types permissive for the virus and an examination of several factors which may affect the growth of the virus in vitro.

MATERIALS AND METHODS

Cell cultures. Two lots of frozen, primary African green monkey (*Cercopithecus aethiops*) kidney (AGMK) cells suitable for use in live-virus vaccine production was obtained from Lederle Laboratories, Pearl River, N.Y., and used as secondary or tertiary cultures. Fetal rhesus kidney cells lines (FRhK-4 and FRhK-6) (19) and continuous AGMK cells (BS-C-1) (8) were kindly provided by H. E. Hopps, Bureau of Biologics, Bethesda, Md. Fetal rhesus monkey lung cells (FRhL-2) (20) were obtained from K. Eckels, Walter Reed Army Institute of Research, Washington, D.C., and continuous owl monkey kidney cells (OMK-210) (4) were provided by C. J. Gibbs, National Institutes of Health, Bethesda, Md. Continuous AGMK cells (CV-1) and fetal rhesus monkey kidney cells (MA-104) were purchased from Microbiological Associates, Bethesda, Md. Human lung carcinoma cells (A-549) were obtained from C. Rapp, U.S. Army Medical Research, Institute of Infectious Diseases, Frederick, Md. (9).

Cell cultures were grown at 35°C in 32-oz (ca. 907-g) glass bottles, 25- to 175-cm^2 plastic flasks, or 490-cm^2 plastic roller bottles containing Eagle minimal essential medium with Earle balanced salt solution, supplemented with 10% heat-inactivated fetal bovine serum (FBS), except where noted. Some cultures required, in addition, either essential or nonessential amino acids and vitamins. MA-104 cells were grown in medium 199 supplemented with 10% heat-inactivated FBS, sodium pyruvate, and nonessential amino acids. Penicillin (100 U/ml) and streptomycin (100 μg/ml) were included in all media.

Reference HAV sera. Pre- and postinfection reference HAV sera were obtained from chimpanzees experimentally infected with HAV MS-1 (14).

HAV. HAV inocula are listed in Table 1. All inocula were prepared in Hanks balanced salt solution, clarified by centrifugation at 7,500 × *g* for 30 min, and passed through a membrane filter (pore size, 0.45 μm). Three inocula were obtained from HAV-infected nonhuman primates. The PA-33 and PA-21 inocula were, respectively, 10% fecal and 1% homogenized liver suspensions prepared from owl monkeys (*Aotus trivirgatus*) naturally infected in Panama (15). The HM-175 inoculum was the gift of S. M. Feinstone, National Institutes of Health, Bethesda, Md., and was a 1% suspension of marmoset liver, representing marmoset passage 6 of this virus strain since its recovery from a human during a hepatitis A outbreak in Australia (3). The remaining seven inocula were all 5 or 10% human fecal suspensions. The FR-AL inoculum came from an outbreak of hepatitis A in Alaska (1). The MS-1 inoculum was prepared from material collected from a human volunteer (day 35) during studies with this virus strain at Joliet, Ill., in 1969 (2). The AH-320 inoculum was a gift from D. Burke, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand, and was collected

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TABLE 1. Detection of HAV antigen on initial passage in primate cell cultures

Inoculum	Mo in culture	Detection of HAV antigen in cell culture[a]:						
		AGMK	BS-C-1	FRhL-2	FRhK-6	FRhK-4	MA-104	OMK-210
PA-33[b]	1	+/ND		−/ND				−/ND
	2	+/+		+/ND				−/ND
	3	+/+		+/+				−/−
PA-21[b]	1		−/−	−/−	Toxic[c]	−/−	−/ ND	−/−
	2		−/+	ND	ND	ND	−/+[d]	ND
HM-175[b]	2	+/ND						
	3	+/+						
FR-AL	1	−/ND	−/ND	−/ND	Toxic			
	2	−/ND	−/ND	−/ND				
	3	+/ND	−/ND	−/ND				
MS-1	1	−/ND		−/ND				−/ND
	2	−/−		−/ND				−/ND
	3	−/−		−/−				−/−
AH-320	1	−/−	−/−	−/−	−/−			
	2	ND	−/−	ND/−	−/−			
	3	ND	−/−	ND/−	+/−[d]			
GR-08	1	+/+	−/−		−/−			
	2	+/+	−/−		−/−			
	3	ND	−/−		−/−			
GR-18	1	ND/ND	−/−		−/−			
	2	+/+	−/−		−/−			
	3	ND/ND	−/−		−/−			
LV-374	1	+/−	−/−		−/−			
	2	+/+	−/−		−/−			
	3	ND	−/−		−/−			
LV-387	1	−/−	−/−		−/−			
	2	+/ND	−/−		−/−			
	3	ND	−/−		−/−			

[a] Solid-phase radioimmunoassay result/direct immunofluorescence result. ND, Not done. All positive results were confirmed in subsequent passages unless otherwise noted.
[b] Nonhuman primate-passaged virus.
[c] Positive for HAV antigen on subsequent passage.
[d] Not confirmed on subsequent passage.

from a sporadic case of hepatitis A occurring in that city during 1981. The GR-08 and GR-18 inocula were obtained from two American soldiers hospitalized during an outbreak of hepatitis A in the Federal Republic of Germany during 1982, and the LV-374 and LV-387 inocula were collected from two ill prisoners during an outbreak of hepatitis A in the U.S. Army Disciplinary Barracks, Leavenworth, Kans., during 1982. All viral inocula contained HAV antigen detectable by solid-phase radioimmunoassay (15), and three inocula (strains PA-33, HM-175, and MS-1) were infectious, as was proven in experiments with various nonhuman primates (2, 3, 11, 14).

Virus isolation and passage. Confluent cell cultures grown in 25- or 75-cm^2 plastic flasks or 5-cm^2 Leighton tubes were washed twice with Hanks balanced salt solution and inoculated with 0.25, 0.50, or 0.15 ml, respectively, of viral inoculum. After a 2-h period of viral adsorption at 35°C, medium containing 2% heat-inactivated FBS was added. The medium was replaced thereafter at 5- to 7-day intervals.

Virus was passed both from the supernatant medium of infected cell cultures and from lysates of the cells themselves. For passage of virus, the culture medium was removed, clarified by centrifugation at 7,500 × *g* for 30 min, divided into aliquots, and frozen at −70°C. Cells were washed twice with Hanks balanced salt solution, mechanically scraped from the surface of the flask, and suspended in a volume of Hanks balanced salt solution representing 20 to 40% of the original volume of maintenance medium. After sonication for 1 min in a cuphorn at 100 W (model W185; Heat Systems-Ultrasonics, Inc., Plainview, N.Y.), the suspension was clarified by centrifugation as described above, divided into aliquots, and frozen at −70°C.

Direct immunofluorescence for HAV antigen. Replication of HAV in Leighton tube cultures was detected by direct immunofluorescence by a method similar to that described previously (3). The immunoglobulin G fraction of a specimen of convalescent-human hepatitis A serum (immune adherence hemagglutination titer, 1:32,000) was purified by a combination of ammonium sulfate precipitation and anion exchange chromatography, as described previously (14), and conjugated to fluorescein isothiocyanate (Bethesda Research Laboratories, Inc., Rockville, Md.) at pH 9.0. Subsequently, conjugated antibody was separated from free fluorescein by passage through Sephadex G-25 (Pharmacia Fine

TABLE 2. Serially propagated HAV strains

HAV strain	Primary isolation	No. of passages (cell culture)	Radioimmunoassay P/N ratio[a]	Results of FA[b]	Infectivity titer[c]
PA-33	AGMK	11 (AGMK)	12.9	+	6.5
PA-33	FRhL-2	5 (FRhL-2)	2.9	+	5.5
PA-21	BS-C-1	7 (BS-C-1)	16.2	ND[d]	6.0
PA-21	FRhK-6	5 (FRhK-6)	6.8	+	5.0
HM-175	AGMK	10 (AGMK) 3 (BS-C-1)	40.3	ND	7.5
FR-AL	AGMK	6 (AGMK)	15.0	+	7.0
GR-08	AGMK	5 (AGMK)	27.4	+	ND
LV-374	AGMK	4 (AGMK)	47.3	+	ND

[a] Solid-phase radioimmunoassay.
[b] FA, Direct immunofluorescence.
[c] Log_{10} HAV titer by in situ radioimmunoassay, radioimmunofocus assay, or direct fluorescence assays.
[d] ND, Not done.

Chemicals, Inc., Piscataway, N.J.). The final fluorescein-antibody conjugate had a fluorescein/protein ratio of ca. 18 μg/mg. Before being stained with the fluorescein conjugate, cell cultures were washed three times with phosphate-buffered saline, pH 7.4, and fixed with acetone for 2 min at room temperature. Cells were overlaid with a 1:4 dilution of the antibody-fluorescein conjugate with rhodamine-albumin counterstain added and allowed to incubate at 35°C for 45 min. Slides were then washed three times with phosphate-buffered saline, mounted under phosphate-buffered saline–glycerol (pH 9.0), and examined immediately with a Dialux 20 fluorescence microscope with a 50-W mercury lamp and an H2 filter block (E. Leitz, Inc., Wetzlar, Federal Republic of Germany). The specificity of the direct immunofluorescence test was confirmed by blocking experiments with paired pre- and postinfection chimpanzee reference sera.

Solid-phase radioimmunoassay for HAV antigen. Cell culture supernatant fluids or cell fractions prepared as described above were tested for HAV antigen by a solid-phase radioimmunoassay carried out in microtiter plates as reported previously (15). Results are shown as the ratio of sample counts per minute (P) to the counts per minute obtained with fresh cell culture medium containing 2% FBS (negative control [N]). P/N ratios equal to or greater than 2.1 were considered positive, but only if they could be blocked more than 50% by the addition of postinfection, but not preinfection, chimpanzee serum (15).

In situ radioimmunoassay for HAV. Synthesis of HAV antigen in infected AGMK or BS-C-1 cell cultures was detected by a modified radioimmunoassay carried out in situ as previously described (13). This method has sensitivity comparable to that of direct immunofluorescence when cultures are held at least 28 days (Binn and Lemon, unpublished data). Titers are reported as 50% tissue culture infective doses per milliliter (18).

Radioimmunofocus assay for titration of HAV. The radioimmunofocus assay, which is based on the immune autoradiographic detection of foci of HAV replication developing under an agarose overlay, was carried out as described previously (13). Results are reported in terms of radioimmunofocus-forming units per milliliter.

RESULTS

Primary isolation of HAV. Ten HAV antigen-positive fecal or liver suspensions, representing seven epidemiologically distinct HAV strains, were inoculated onto a variety of primate cell cultures (Table 1). Of the cells tested, AGMK cells appeared to be most permissive for the virus. Intracellular viral antigen was most frequently identified by either solid-phase radioimmunoassay or direct immunofluorescence in AGMK cell cultures. Positive slides demonstrated typical, granular, cytoplasmic HAV fluorescence, as described by others (3, 17). Three of the nine specimens inoculated onto AGMK cells were positive at 1 month, six were positive by 2 months, and one was positive after 3 months. The proportion of cells demonstrating positive fluorescence increased gradually with time after inoculation and eventually approached 100% with most isolates. Two inocula (strains MS-1 and AH-320) did not give any evidence of replication in AGMK cells, although the cultures of strain AH-320 were terminated after only 30 days for technical reasons. In contrast, the continuous BS-C-1, FRhL-2, and FRhK-6 cell lines appeared to be less permissive for HAV, as viral antigen was identified in these cells less often or only after longer latent periods. No evidence for production of viral antigen was noted in the OMK-210 cell line.

All three inocula derived from nonhuman primate-passaged material (strains PA-33, PA-21, and HM-175) were readily recovered in cell cultures. Viral replication was noted in BS-C-1 and FRhL-2 cells only when they were inoculated with primate-passaged material. In general, virus was successfully isolated directly from human specimens only in AGMK cells, although minimal antigen was detected by radioimmunoassay (not immunofluorescence) 3 months after the inoculation of FRhK-6 cells with strain AH-320. No cytopathic effect was apparent in any of these cell cultures.

Serial propagation of HAV. Serial propagation of virus was achieved with most primary isolates (Table 2). In addition to cell-associated viral antigen, after passages 3 to 5, HAV antigen could be detected readily by solid-phase radioimmunoassay in the supernatant media of cell cultures infected with each of these viruses. The early passage history of strain HM-175 is shown in Table 3. Virus was initially harvested 2 months after inoculation with the original marmoset liver suspension, at which time a cell lysate was minimally reactive by radioimmunoassay (P/N ratio, 2.4). Subsequently, virus was harvested and passed every 14 to 30 days. By passage level 5, viral antigen could be detected in supernatant fluids 14 to 28 days after inoculation. With succeeding passages, generally increasing quantities of viral antigen were noted in both cell and supernatant fractions (Table 3). After 10 passages in AGMK cells, this strain was adapted to growth in BS-C-1 cells. Viral titrations carried out by in situ radioimmunoassay on cell and supernatant fractions at passage levels 11 and 12 (passage levels 1 and 2 in BS-C-1 cells) indicated that approximately equal titers of virus were present in each fraction (10^7 to 10^8 50%

TABLE 3. Serial propagation of HAV HM-175 in AGMK cells

Passage no.	Day harvested	Radioimmunoassay P/N ratios for HAV antigen in[a]:	
		Supernatant fluid	Cell fraction
1	60	ND	2.4
2	30	ND	6.2
3	14	0.7	2.7
4	21	ND	0.5
5	28	3.3	7.3
6	21	3.2	6.6
7	20	2.3	6.6
8	21	5.4	14.5
9	21	2.7	10.2
10	21	14.8	21.2

[a] Solid-phase radioimmunoassay P/N ratio. All values >2.1 demonstrated specific blocking (>50%) with reference HAV sera. ND, Not done.

tissue culture infective doses per milliliter). No cytopathic effect was apparent in these cultures.

Survey of primate cell cultures for ability to support HAV replication. To ascertain the range of permissiveness among a variety of primate cell cultures, eight different cell cultures were simultaneously infected with fifth-passage HAV PA-33 grown in AGMK cells, and cells were examined periodically by direct immunofluorescence (Table 4). In general, these results were similar to those obtained during attempts at primary isolation of virus. Fluorescence was first noted in AGMK cells at day 5 and increased markedly during the ensuing week. However, strong fluorescence was also noted in both FRhK-6 and BS-C-1 cells by 19 days after inoculation, indicating that these cells were moderately permissive for HAV. Although some degree of fluorescence was noted in most of the other cell cultures tested, such fluorescence either was minimal or did not develop until very late. No evidence of viral antigen synthesis was noted in the A-549 human lung carcinoma cells.

Effect of temperature on synthesis of HAV antigen in BS-C-1 cells. Tube cultures of BS-C-1 cells were infected with 10-fold dilutions of a strain PA-21 virus seed (passage six), held at 32, 35, or 39°C, and tested at 7-day intervals by in situ radioimmunoassay for development of viral antigen. Results (Table 5) indicated that 35°C (the temperature at which strain PA-21 was originally adapted to cell culture) was optimum. Viral antigen was detected at day 14 in cells infected at 35°C with a dilution of the virus seed which was 1000-fold less than that required to demonstrate antigen synthesis at 32 or 39°C.

Production of HAV in serum-free media. We investigated parameters influencing the production of viral antigen and the final virus yield in 490-cm^2 roller flask cultures of BS-C-1 cells. Flasks were infected at an estimated multiplicity of 0.1 per cell and, after an absorption period of 2 h, were fed with either 50 ml of standard maintenance medium (Eagle minimal essential medium with 2% FBS) or medium 199 without serum. Supernatant fluids were replaced at weekly intervals and assayed for viral antigen by solid-phase radioimmunoassay and for titer of infectious virus by in situ radioimmunoassay. The inoculum virus was strain PA-21 (passage five), originally isolated in BS-C-1 cells. After 7 days, consistently higher radioimmunoassay P/N ratios and infectivity titers were achieved in the supernatant fluids of cells infected in the absence of serum and maintained in medium 199. Similarly, higher virus yields were found in serum-free cells when the cell monolayer was mechanically removed, sonicated, and tested for viral antigen at the conclusion of the experiment (Table 6). Subsequent experiments with cell culture-adapted strain HM-175 confirmed these findings (data not shown).

In preliminary experiments, it was noted that the use of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer in medium 199 appeared to result in a reduction in the amount of viral antigen released into supernatant fluids (B. Innis, personal communication). This reduction was studied in detail in BS-C-1 cells infected with strain HM-175 (Fig. 1). In this experiment, viral antigen first appeared in supernatant culture fluids 14 days after inoculation but was present at consistently lower concentrations (as reflected in solid-phase radioimmunoassay P/N ratios) in the presence of 25 mM HEPES. These differences, however, were not reflected in the titer of virus released into the supernatant fluid or contained in the cells at the time of harvest (Fig. 1). Similar results were obtained with BS-C-1 cells infected with strain PA-21 (passage 7) in medium 199 in the presence or absence of HEPES buffer (data not shown).

The apparent inhibitory effect of HEPES buffer could not be related to direct interference in the solid-phase radioimmunoassay system, because addition of up to 100 mM HEPES buffer to samples containing HAV antigen resulted in a reduction in the P/N ratio obtained of less than 15%. Together, these results suggest that the addition of HEPES buffer to serum-free media results in a moderate inhibition of release of viral antigen (which is predominantly noninfectious) from infected BS-C-1 cells.

DISCUSSION

Although the propagation of HAV in cell culture was a very significant achievement, further technical improvements are required to facilitate diagnostic methods based on recovery of virus, biochemical characterization of the virus, or attempts at vaccine development. We therefore attempted to grow several different strains of HAV in known susceptible cells as well as in other cell lines possessing potentially more desirable characteristics. All the isolates recovered were noncytopathic and all produced persistant infections. The pattern of immunofluorescence observed was highly characteristic, similar for each strain, and similar to that described previously (3, 17). The identity of each isolate was verified at every passage level by blocking tests in the solid-phase radioimmunoassay.

For initial recovery of the virus, the AGMK cells proved most useful. The relative lack of success we experienced with FRhK-6 cells for primary isolation stands in contrast to

TABLE 4. Detection of HAV antigen in primate cell cultures inoculated with HAV PA-33[a]

Cell	Detection of HAV antigen on day postinoculation[b]:				
	5	12	19	26	35
AGMK	(+)	3+	4+	4+	4+
FRhK-6	−	+	3+	3+	ND
BS-C-1	−	−	4+	4+	4+
FRhK-4	−	−	(+)	+	3+
FRhL-2	(+)	−	ND	+	ND
CV-1	−	−	−	(+)	+
MA-104	−	−	−	−	2+
A-549	−	−	−	−	−

[a] Approximately 10^4 virus from passage 5 grown in AGMK cells. Uninoculated cultures did not develop any fluorescence.

[b] Direct immunofluorescence scale: −, (+), +, 2+, 3+, 4+. ND, Not done.

TABLE 5. Effect of temperature on synthesis of viral antigen in BS-C-1 cells infected with HAV PA-21

Temp (°C)	Result on day postinoculation[a]:			
	7	14	21	28
32	1.5	1.5	3.5	3.5
35	1.5	4.5	5.5	5.5
39	1.5	1.5	3.5	5.0

[a] Reciprocal log of highest inoculum dilution yielding positive results in the in situ radioimmunoassay as estimated by the method of Reed and Muench (18).

earlier studies in which this cell line proved suitable for this purpose (16). This may relate to the higher passage levels (15 to 20) of FRhK-6 cells used in our study. The AGMK cells produced relatively large quantities of viral antigen which were readily detected in solid-phase radioimmunoassay and immunofluorescence tests. In previous studies (3, 17), however, the presence of adventitious agents in AGMK cells had limited their use. This problem was eliminated by the use of frozen, pretested, certified AGMK cells which were free of contaminating, adventitious agents. After five passages in these cells, the strain PA-33 isolate was readily propagated in several AGMK and fetal rhesus monkey cell cultures (Table 4). This finding suggests that the virus had developed a wider host range in primate cell cultures after initial adaptation to AGMK.

The limited supply of certified AGMK cells, however, eventually necessitated trials of AGMK cells from other sources and the evaluation of other cell lines for viral growth characteristics. In our experience, however, commercially acquired AGMK cells often developed cytomegalovirus-like cytopathic effects which severely limited their usefulness (Binn, unpublished data). Such problems might be overcome by careful selection and subsequent use of a single lot of frozen, commercially available AGMK cells. Of the continuous cell lines examined, the BS-C-1 cells appeared most suitable for further use because of their relative permissiveness for cell culture-adapted HAV and their excellent growth characteristics. Although HAV PA-21 was successfully isolated from an owl monkey liver specimen directly in BS-C-1 cells, this cell line was not generally suitable for primary isolation (see Table 1). Nonetheless, strains PA-33 and HM-175 were both readily grown in these cells after initial recovery in AGMK cells. The BS-C-1 cell line is derived from normal AGMK cells and has been extensively used for the propagation of a wide spectrum of viruses (8). These cells are readily available and free from detectable adventitious agents, and they can be grown in quantity. Furthermore, they can be maintained with comparatively simple media for long periods.

TABLE 6. Production of HAV PA-21 in BS-C-1 cells grown in roller flasks

Culture fraction	Growth period (days)	P/N ratio of HAV antigen on:		HAV $TCID_{50}$s per milliliter[a]	
		EMEM 2% FBS[b]	M199[c]	EMEM 2% FBS	M199
Supernatant medium	7	3.8	3.8	6.0	5.5
Supernatant medium	14	16.3	20.8	5.5	6.5
Supernatant medium	21	10.8	35.3	6.0	6.5
Cell fraction	21	19.8	32.1	5.5	6.5

[a] HAV titer determined by in situ radioimmunoassay. $TCID_{50}$, 50% tissue culture infective dose.
[b] Eagle minimal essential medium with 2% FBS.
[c] Medium 199 without FBS.

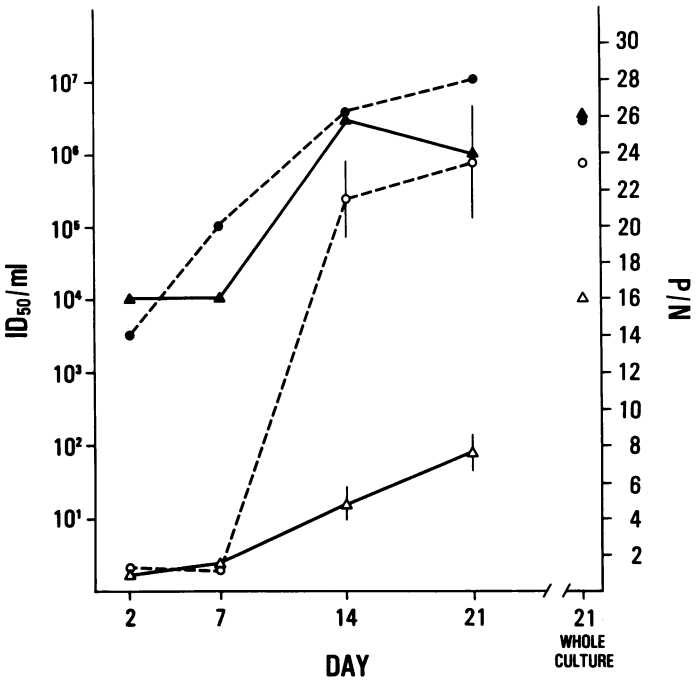

FIG. 1. Production of HAV antigen and infectious virus in roller bottle cultures of BS-C-1 cells infected with cell culture-adapted strain HM-175 virus and maintained in the presence or absence of 25 mM HEPES buffer. Symbols: ○, △, radioimmunoassay results (P/N ratios); ●, ▲, results of viral titrations; ————, cultures with HEPES buffer; — — —, cultures maintained in the absence of HEPES buffer. Whole culture, Results achieved with cells disrupted in 50 ml of supernatant fluid.

Groups of investigators have reached different conclusions concerning the release of HAV or viral antigen from infected cultures into supernatant fluids (3, 5–7). In our studies, each of the recovered viruses could be detected by solid-phase radioimmunoassay in supernatant fluids after passages 3 to 5. With both the solid-phase radioimmunoassay and the in situ radioimmunoassays, we observed that the presence of serum in the maintenance media was not necessary for viral propagation or release of virus into the supernatant fluids. In fact, cultures maintained in serum-free media had solid-phase radioimmunoassay values that were approximately 2-fold higher and infectivity titers that were 3- to 10-fold higher (Table 6). Thus, it is possible to prepare viral antigen from infected cells maintained in serum-free media, which may be advantageous both for antiserum preparation and for potential vaccine production. In recent tests, such cell culture preparations have proven to be immunogenic for both guinea pigs and rabbits (L. N. Binn, R. H. Marchwicki, S. M. Lemon, N. L. Gates, H. G. Cannon, and W. H. Bancroft, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 23rd, Las Vegas, Nev., abstr. no. 2, 1983).

The virus strains recovered from owl monkeys (15) require further comment. Antigenic studies carried out by solid-phase radioimmunoassay (15) and virus cross-neutralization methods (12) indicate that these viruses are indistinguishable from recognized human strains of HAV. Such studies have strengthened epidemiological observations which suggest that strains PA-21 and PA-33 were originally of human origin (15).

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