PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 167, 201–206 (1981)

Isolation of Hepatitis A Virus *in Vitro* in Cell Culture Directly from Human Specimens (41149)

PHILIP J. PROVOST, PAULA A. GIESA, WILLIAM J. McALEER, AND MAURICE R. HILLEMAN

Division of Virus & Cell Biology Research, Merck Institute for Therapeutic Research, West Point, Pennsylvania 19486

Abstract. Human hepatitis A virus (HAV) was isolated directly in cell culture from infected human fecal extracts using techniques developed for *in vitro* cultivation of the marmoset-adapted CR326 strain of human HAV. A very long incubation was required in first passage, but vigorous cell culture growth, with incubation periods and virus yields similar to those of the CR326 strain that was isolated originally in marmosets, was achieved on serial passage. As with the CR326 strain, there were no evident cytopathic effects. The isolates proved to be HAV virus based on specific activity in serologic tests and by tests for infectiousness in marmosets.

Primary viral hepatitis is caused principally by two viruses, designated hepatitis A and hepatitis B. A major breakthrough in studies of human hepatitis A was recorded in a recent report (1) from these laboratories describing the propagation of the hepatitis A virus in cell culture. The CR326 strain virus that was propagated had been passaged in marmosets and the source of virus was infected liver of these animals. The present report describes the direct propagation in cell culture of virus from infected stools of human hepatitis A cases. These findings are of importance in showing that marmoset passage is not mandatory for *in vitro* propagation of the agent and in providing virus for vaccine development that is free of prior marmoset passage that might affect attenuation characteristics.

Materials and Methods. *Human fecal extracts.* Three human fecal extracts were tested. The first, fecal extract (FE) 2-23-31-44, was a pool of specimens from four acutely ill Costa Rican patients. Prior to pooling, each fecal specimen was homogenized with phosphate-buffered saline (PBS) for 1 min at full speed in a Sorvall Omni-Mixer and was then clarified at 10,000 rpm for 30 min using a Beckman SW30 rotor. The pooled 20% fecal extracts had an HAV antigen titer of 1:16 when assayed by the immune adherence hemagglutination test (IAHA). Extracts were also made of feces from Costa Rican patient 033-03. For these, stool samples were mixed with 4 vol of PBS, homogenized by shaking with glass beads, and clarified by centrifugation at 6000 rpm for 1 hr. One extract, FE 033-03, 0, was made from feces obtained on the day of onset of hepatitis and the other, FE 033-03, 7, was prepared from feces obtained on the seventh day after onset of clinical disease. FE 033-03,0 gave a negative test for HAV antigen by IAHA, but was positive by radioimmunoassay (RIA) (P/N = 5.6). FE 033-03,7 gave negative tests for HAV antigen in both assays. *Cell cultures.* Fetal rhesus kidney cell cultures (FRhK6) (2) were grown and maintained as described previously (1), with the exception that the penicillin and streptomycin were replaced by neomycin in a concentration of 50 μg/ml.

Virus propagation. The fecal extracts were diluted 1:5 in cell culture maintenance medium, filtered through 0.45-μm Millex filters, and 1-ml amounts were absorbed for 4 hr at 35° to the confluent sheets of FRhK6 cells in 25-cm^2 flasks. The cultures were drained, refed with 5 ml of maintenance medium, and incubated at 35° with medium changes at 4- to 5-day intervals. Growth of HAV was monitored by direct immunofluorescence (IF) performed on acetone-fixed cell monolayers on coverslips removed at periodic intervals (1). When evidence for HAV antigen was detected by immunofluorescence, the cell cultures were harvested by freezing and thawing twice and the sus-

0037-9727/81/060201-06$01.00/0

pension was sonicated (1). The second passage was carried out in FRhK6 cells in 25-cm^2 flasks using 1 ml of the first passage virus, diluted 1:2, as inoculum. All subsequent passages of FE 2-23-31-44 and FE 033-03,0 viruses were made using 1 ml of 1:5 and 1:20 dilutions of the preceding passage material, respectively. RIA and IAHA assays were performed on extracts prepared from cultures harvested at optimal times as determined by the IF assay.

Assays. The direct IF test for HAV antigen was performed as described earlier (1), using fluorescein-conjugated anti-HAV human globulin at 1:100 dilution in PBS. This assay measured presence of hepatitis A virus antigen in infected cells treated with fluorescein-conjugated hepatitis A antibody. Immunologic identification of the antigen in the infected cells as hepatitis A virus was accomplished by the IF blocking assay. In the tests, one part of preillness or one part of convalescent serum from human hepatitis A patient 109-06 was mixed with four parts of diluted conjugate prior to application to the cells. Lack of fluorescence in tests with the convalescent serum specimen indicated presence of antigen identifiable as hepatitis A. The IAHA assays for HAV antigen and anti-HAV were carried out as reported earlier (3). In the test for antigen, freeze–thaw extracts of infected cell cultures were assayed for ability to fix complement in the presence of known homologous antibody, causing aggregation of human type O red blood cells. The same assay was used to measure antibody in tests in which several hepatitis A antigens were employed. The RIA for hepatitis A antigen in cell culture extracts was carried out as described earlier (1) and the RIA for anti-HAV was done by HAVAB assay (Abbott). In the RIA assay for antigen, the radioactive counts of the test materials were divided by the average count for five negative control samples and the results were expressed as the *P/N* (positive/negative) ratio. Immune electron microscopic examination of extracts of infected cell cultures was carried out by ordinary procedures (4). Marmoset infectivity was measured by intravenous inoculation of *Saguinus labiatus* marmosets with 1 ml of a 1:10 dilution of cell culture harvest, with subsequent measurement of serum enzymes and anti-HAV responses (1, 4, 5).

Results. Table I summarizes the findings in studies of the propagation of human HAV from FE 2-23-31-44 in FRhK6 cell cultures. In the first passage, suggestive evidence for development of immunofluorescent, intracytoplasmic granules characteristic of HAV infection (1) was noted 33 days after inoculation. The cultures were harvested on Day 37 and the product was only weakly positive in the RIA for HAV

TABLE I. CULTIVATION OF HUMAN HEPATITIS A VIRUS DERIVED FROM FECAL EXTRACT 2-23-31-44 IN SERIAL PASSAGE IN FRhK6 CELL CULTURES

	HAV content at serial cell culture passage level[a]					
	1	2	3	4	5	6
Day of Harvest	37	19	20	14	14	14
HAV content estimated by:						
Immunofluorescence[b,c]	±	+	4+	4+	4+	4+
Immune adherence	<1	<1	<1	1	2	<1
Radioimmunoassay (P/N)	1.8	1.8	16.0	20.0	31.2	26.4
Marmoset inoculation[d]	N.D.	N.D.	N.D.	N.D.	Infectious	N.D.

[a] Serially passaged control cultures (not inoculated with virus) were tested identically to the virus cultures and were uniformly negative.

[b] Degree of immunofluorescence: ± indicates suggestive fluorescent granules in <25% of cells; + indicates fluorescent granules present in ~25% of cells; 4+ indicates heavy levels of fluorescent granules in greater than 75% of cells.

[c] At serial passages 3 through 6, immunofluorescence was specifically blocked by unlabeled human convalescent hepatitis A serum and not by preillness serum of the same patient.

[d] Two of two marmosets injected with passage 5 harvest developed hepatitis, one with enzyme elevation at Day 20, the other at Day 27. Both also developed anti-HAV by HAVAB and IAHA. N.D. = not done.

TABLE II. CULTIVATION OF HUMAN HEPATITIS A VIRUS DERIVED FROM FECAL EXTRACT 033-03,0 IN SERIAL PASSAGE IN FRhK6 CELL CULTURES

	HAV content at serial cell culture passage level[a]									
	1	2	3	4	5	6	7	8	10	20
Day of harvest	63	16	13	13	13	13	11	9	9	9
HAV content estimated by:										
Immunofluorescence[b,c]	+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Immune adherence	<1	2	1	1	1	2	2	1	1	2
Radioimmunoassay (P/N)	3.6	21.3	20.0	18.1	16.5	18.0	17.2	10.2	11.8	25.0
Marmoset inoculation[d]	N.D.	N.D.	N.D.	N.D.	Infectious	N.D.	N.D.	N.D.	Infectious	N.D.

[a] Serially passaged control cultures were tested identically to the virus cultures and were uniformly negative.

[b] Degree of immunofluorescence: + indicates fluorescent granules in about 25% of cells; 4+ indicates heavy levels of fluorescent granules in greater than 75% of cells.

[c] At serial passages 2 through 20, immunofluorescence was specifically blocked by unlabelled human convalescent hepatitis A serum and not by preillness serum of the same patient.

[d] Four of four marmosets inoculated with the 5th passage harvest developed hepatitis. One animal developed elevated enzymes at Day 13, two at Day 20, and one at Day 27. All developed anti-HAV by HAVAB and IAHA. Four of four marmosets inoculated with 10th passage virus also developed hepatitis. One animal developed elevated enzymes at Day 22 and the remaining three at Day 29. All developed anti-HAV by HAVAB and IAHA. N.D. = not done.

antigen. On second passage, immunofluorescent granules were detected earlier and the cultures were harvested on Day 19. Again, the product gave only a weakly positive RIA for HAV antigen. On subsequent passages, the virus yields increased and the incubation periods decreased consistent with our previous experience in cultivation of HAV (1). At passages 3 through 6, immunofluorescence was specifically blocked by human convalescent hepatitis A serum but not by preillness serum of the same patient. The passage 5 harvest fluid produced serum isocitric dehydrogenase enzyme elevations in 2/2 marmosets (one at Day 20, one at Day 27) followed by strong HAVAB and IAHA anti-HAV responses.

Uninfected cell cultures were prepared and maintained throughout for control purpose, and were found negative for HAV in tests carried out in the same way as for the virus-infected materials.

Table II summarizes the findings in the

FIG. 1. Photomicrograph of hepatitis A virus-infected FRhK6 cells stained by direct immunofluorescence at Day 9 of the 14th serial passage of FE 033-03,0 in FRhK6 cells. Numerous cytoplasmic fluorescent (apple green) granules are evident.

studies of the propagation of human HAV from FE-033-03,0 in FRhK6 cell cultures. In the first passage, intracytoplasmic immunofluorescent granules were first detected on Day 53 after inoculation and the cultures were harvested on Day 63. The product gave only a weak RIA assay for HAV antigen. By contrast, comparable studies of FE 033-03,7, in the first passage, gave entirely negative results for HAV antigen by IF and RIA. Further passages were not attempted with FE 033-03,7. The HAV strain derived from FE-033-03,0 was adapted rapidly to growth in FRhK6 cells as evidenced by a marked decrease in incubation time and increase in virus yield at the second passage. Subsequent passages were consistent with our prior experience (1) in terms of incubation periods and virus yields on adaptation of HAV to cell culture. IF was specifically blocked at passages 2 through 20. Throughout these serial passages of FE 033-03 as well as those of HAV strain FE 2-23-31-44, no obvious cytopathic effects of virus growth were noted in the FRhK6 cell cultures. This too is consistent with previous observations (1). Passage 5, 10, and 15 harvest fluids induced serum isocitric dehydrogenase enzyme elevations in all of four marmosets per passage and the animals showed strong HAVAB and IAHA anti-HAV responses. Figure 1 shows the typical immunofluorescence appearance of an infected cell culture at passage 14 of virus from FE 033-03,0. Figure 2 shows a typical electron micrograph of passage 20 of the virus from FE 033-03,0, aggregated with specific immune serum.

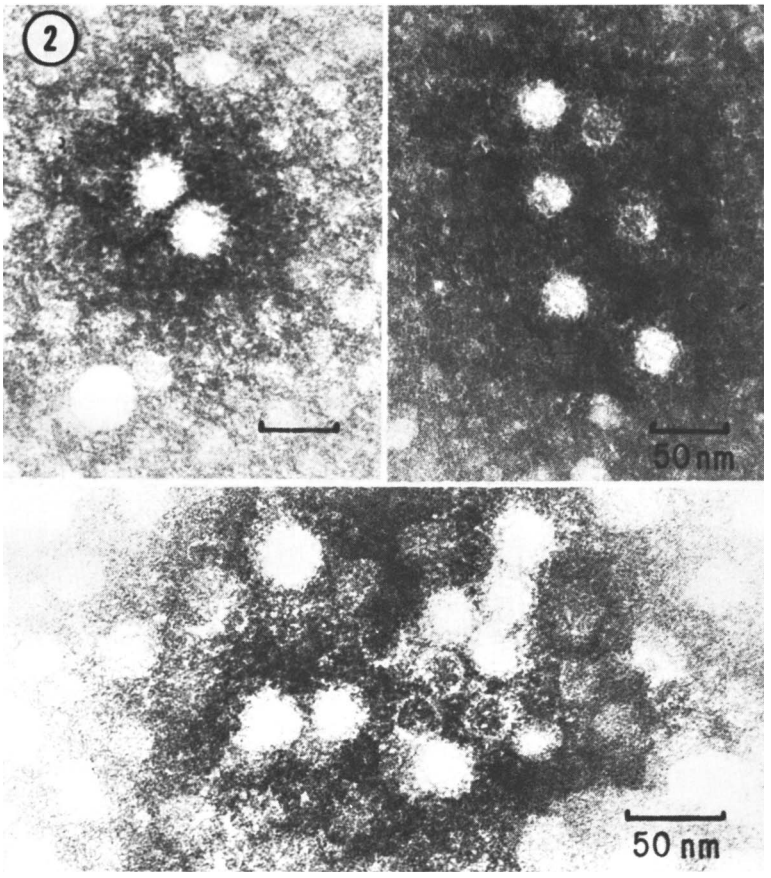

FIG. 2. Electron micrograph of hepatitis A virus particles, as detected in an extract of the 20th serial passage of FE 033-03,0 in FRhK6 cells, reacted with convalescent serum of pt. 109-06. Phosphotungstic acid stained.

Table III gives the results of tests comparing the antigenic specificity in IAHA testing for anti-HAV of conventional HAV antigen derived from marmoset liver and three HAV antigens derived from FRhK6 cell cultures. Two of the cell culture-derived antigens were from the fecal extract isolates of the present study, and the other was derived from the marmoset-passaged CR326 strain of HAV (1). All four HAV antigen preparations behaved similarly in their reaction with anti-HAV and their lack of reactivity with known seronegative human sera.

Discussion. We previously described the *in vitro* propagation of HAV in cell culture (1), using as inoculum an extract of HAV-infected marmoset liver from the 31st passage *in vivo* of the CR326 strain of HAV in marmosets. This liver extract, used as inoculum in FRhK6 cells, infected cell cultures as evidenced by direct immunofluorescence within the first week after inoculation and extensive virus development within a 3-week incubation period. This was in distinct contrast to the present findings with human fecal extract inocula in which HAV growth in the first passages in cell culture could not be detected by direct immunofluorescence until 5 to 8 weeks after inoculation. It is clear that prior marmoset passage of HAV and the use of liver extract as inoculum increased the ability to isolate HAV in cell culture. Were it not for the knowledge obtained from the cultivation of the marmoset-passaged CR326 strain of HAV, we probably would not have been able to isolate HAV in cell culture from human fecal extracts. A similar enhancement of *in vitro* growth capacity by animal passage has recently been reported (6) for human rotavirus type 2. Although the adaptation of the fecal-derived HAV strains was slow on initial passage, these viruses adapted rapidly and grew vigorously in subsequent serial passages comparable to that of the marmoset-passaged CR326 virus. A preliminary report of an independent isolation of HAV in cell culture from human stool was made recently (7).

Since the HAV strain isolated from FE 2-23-31-44 was derived from a pool of fecal extracts from four patients, we chose to discontinue serial passage of the mixture in favor of concentrating our effort on the serial passage of the HAV strain derived from patient 033-03. This strain of HAV was originated from the same disease episode of

TABLE III. COMPARABLE SPECIFICITY OF MARMOSET LIVER-DERIVED AND CELL CULTURE-DERIVED HEPATITIS A ANTIGENS IN THE IMMUNE ADHERENCE HEMAGGLUTINATION ASSAY FOR HEPATITIS A ANTIBODY

Subjects	Case no.	Time of specimen (Days)	Antibody Titer vs. Antigen from: Marmoset Liver, CR326[a]	Cell culture CR326[b]	Cell culture FE 2-23-31-44[c]	Cell culture FE 033-03, 0[d]
Hepatitis A	033-02	−21	<5	<5	<5	<5
		+36	≥2560	≥2560	≥2560	≥2560
		+102	≥2560	≥2560	≥2560	2560
Hepatitis B	039-01	−108	<5	<5	<5	<5
		+10	<5	<5	<5	<5
		+149	<5	<5	<5	<5
Normal human sera	2		160	640	640	320
	16		<5	<5	<5	<5
	20		<5	<5	<5	<5

[a] Tests performed 3/76 using standard CsCl-purified hepatitis A antigen derived from *in vivo* infected marmoset liver (5).

[b] Tests performed 7/78 using fifth passage harvest of the CR326 strain in FRhK6 cell culture (1). The CR326 strain had been passed 31 times *in vivo* in marmosets prior to cell culture passage.

[c] Tests performed using fifth passage harvest of the HAV strain derived from FE 2-23-31-44 in FRhK6 cell culture.

[d] Tests performed using sixth passage harvest of the HAV strain derived from FE 033-03,0 in FRhK6 cell culture.

the same patient whose blood yielded the CR326 strain of HAV (4, 8) in marmosets and was subsequently grown in cell culture (1). Evidence for the stability of infectivity of HAV on prolonged storage is provided by the fact that the fecal material used in the present study had been stored at −70° since 1967.

The Day 7 postonset fecal extract from patient 033-03 gave negative findings in cell culture through 63 days, a time at which the Day 0 extract was positive. This suggests decline in viral excretion in hepatitis A cases soon after onset of clinical symptoms.

Though not reported here, we also attempted to isolate HAV in cell culture from six human hepatitis A serum specimens, all taken within a 7-day period preceding onset of clinical disease. We used low passage newborn cynomolgus monkey kidney as cell culture substrate. Studies with cell culture-adapted HAV in our laboratory had established that these cells were similar to FRhK6 in their capacity to support HAV growth. All six sera gave negative results for HAV antigen in cell culture through a 10-week incubation period. Evidently, the amount of virus, if any, in the human serum was insufficient to permit direct isolation in cell culture.

We thank Frank Banker, B. S., W. P. MacClure Fisher, B. S., William Hurni, M. A., Marilyn Johnston, B. S., and Paul Koser, B. S., for expert technical assistance, William Miller, M. S., for devising the radioimmunoassay for hepatitis A antigen, and Bohdan S. Wolanski, Ph.D., for performing electron microscopy. Human clinical specimens were kindly provided by Victor M. Villarejos, M.D.

1. Provost, P. J., and Hilleman, M. R., Proc. Soc. Exp. Biol. Med. **160**, 213 (1979).
2. Wallace, R. E., Vasington, P. J., Petricciani, J. C., Hopps, H. E., Lorenz, D. E., and Kadanka, Z., In Vitro **8**, 333 (1973).
3. Miller, W. J., Provost, P. J., McAleer, W. J., Ittensohn, O. L., Villarejos, V. M., and Hilleman, M. R., Proc. Soc. Exp. Biol. Med. **149**, 254 (1975).
4. Mascoli, C. C., Ittensohn, O. L., Villarejos, V. M., Arguedas G., J. A., Provost, P. J., and Hilleman, M. R., Proc. Soc. Exp. Biol. Med. **142**, 276 (1973).
5. Provost, P. J., Villarejos, V. M., and Hilleman, M. R., Proc. Soc. Exp. Biol. Med. **155**, 283 (1977).
6. Wyatt, R. G., James, W. D., Bohl, E. H., Theil, K. W., Saif, L. J., Kalica, A. R., Greenberg, H. B., Kapikian, A. Z., and Chanock, R. M., Science **207**, 189 (1980).
7. Frösner, G. G., Deinhardt, F., Scheid, R., Gauss-Müller, V., Holmes, N., Messelberger, V., Siegl, G., and Alexander, J. J., Infection **7**, 303 (1979).
8. Provost, P. J., Ittensohn, O. L., Villarejos, V. M., Arguedas G., J. A., and Hilleman, M. R., Proc. Soc. Exp. Biol. Med. **142**, 1257 (1973).

Received November 24, 1980. P.S.E.B.M. 1981, Vol. 167.