

Propagation of Human Hepatitis A Virus in Cell Culture *in Vitro* (40422)

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Human hepatitis A virus, like that of hepatitis B, has defied reliable propagation *in vitro* in the laboratory for more than a half century (1). Deinhardt *et al.* (2) provided the first evidence for propagation of hepatitis A virus in marmosets and, following this lead, we recovered the CR326 strain of human hepatitis A virus in *Saguinus mystax* marmosets (3). Employing virus obtained from infected marmoset serum and liver, we gave (4) precise definition to the physical and chemical attributes of the virus that we described as an enterovirus-like agent, and developed serum neutralization (5), complement-fixation (6), and immune adherence (7) assays for hepatitis A virus and antibody. The development of a means for measuring antibody made possible serodiagnostic and seroepidemiologic investigations (8) of the disease and the standardization of human immune globulin for use in prophylaxis in man. Recently, we described the susceptibility of the *S. labiatus* (rufiventer) marmoset to the virus (9) and the preparation of an inactivated virus vaccine (10) using virus that was purified from infected rufiventer marmoset liver. The vaccine induced antibody and protected marmosets against challenge with the agent.

We have now been able to propagate the CR326 strain in liver explant cell cultures of *S. labiatus* marmoset and in a fetal rhesus kidney normal cell line. The findings are reported here.

Materials and methods. *Human hepatitis A virus.* CR326 human hepatitis A virus passed 5 times in *S. mystax* and 26 times in *S. labiatus* marmosets (10) was employed. The initial seed stock was a 4% suspension of infected liver in phosphate buffered saline (PBS) that was clarified by low speed centrifugation, filtered through an 0.45 μ Millipore filter, and stored frozen. The seed proved infectious for *S. labiatus* on intravenous injection.

S. labiatus cell culture. Finely minced frag-

ments of *S. labiatus* liver were washed in 20% fetal calf serum in culture medium and were planted in 25 cm² Falcon flasks, some of which contained coverslips. The medium consisted of Williams medium E (Flow Laboratories) containing 20% normal marmoset serum, 10⁻⁶ M glucagon, 2 mM glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin used in 2 ml amounts with 3x weekly refeds. The atmosphere was 5% CO₂ in air and incubation was on a rocker platform at 35°. Hepatocyte-like epithelial cell outgrowth was evident by the 7th day, and the cultures were maintained for many weeks. Cells were maintained in the same medium during viral propagation.

Fetal rhesus kidney (FRhK6) cell cultures. The FRhK6 cell line was originated by Wallace *et al.* (11) from the kidney of a fetal rhesus monkey. Little is known about the line other than that the cells are epithelial-like and that the maximum population doubling is 12. This line (11) was obtained from Dr. John Petricciani, Bureau of Biologics, FDA, in passage 3, and frozen seed stock was prepared at passage 5. Cultivation was in 25 cm² Falcon flasks, some of which contained coverslips. The growth medium was Williams medium E containing 20% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin, and the maintenance medium was Eagle's minimal essential medium containing 0.5% fetal calf serum, glutamine, penicillin and streptomycin as above. The cultures were gassed with 5% CO₂ in air and were incubated at 35° on a rocker platform. Medium changes were made 1-2x per week.

Virus propagation. The cultures in 25 cm² flasks were drained, washed with PBS, and inoculated with 0.2 ml of virus in 0.4 ml of medium, and absorption was allowed to proceed for 4 hr after which 1.4 ml or 4.4 ml (passages 2-8 of virus in FRhK6) maintenance medium was added and incubation

was carried out at 35° with 5% CO₂ in air. Coverslips were examined twice weekly by immunofluorescence for presence of hepatitis A virus antigen. The cultures in the flasks were harvested after two freeze-thaw cycles, sonicated for 15 sec, clarified by low speed centrifugation, and stored frozen until tested. Uninoculated cell cultures for control purpose were prepared and handled identically.

Direct immunofluorescence assay for detecting antigen. The coverslip cultures were rinsed in PBS and fixed in acetone. Methods were similar to those of Mathiesen *et al.* (12), but developed independently. Human hepatitis A globulin was prepared by fractionation of convalescent serum from documented cases of the disease, and this was conjugated with fluorescein isothiocyanate. The conjugate was absorbed twice with acetone-dried normal marmoset liver powder prior to use and used at 1:75 dilution in PBS. Examination was made using a Zeiss microscope with an epi-fluorescence condenser, a 63X planapochromatic objective, and appropriate filters.

Immunofluorescence blocking assay for hepatitis A antibody. Certain of the cultures were tested with paired sera from 2 patients with hepatitis A infection for purpose of establishing identity of the antigen and specificity of the test. The hepatitis A immune adherence (IA) titers of the sera were <1:5 preillness and ≥1:2560 post-illness. In the tests, the sera at 1:5 dilution were applied to the coverslips prior to addition of conjugate and observations were made for prevention of immunofluorescence.

Serum neutralization tests in vitro. Hepatitis A virus preparations passed 4 times in liver cell cultures and 5 times in FRhK6 cell cultures, at 1:10 and 1:100 dilutions respectively, were incubated with the paired human hepatitis A sera at 1:5 dilution, and then 0.2 ml inoculated onto the corresponding cell cultures. Coverslip cultures were examined by direct immunofluorescence through 21 days after inoculation for presence of human hepatitis A virus antigen.

Virus infectivity titration. Harvests of the fifth and sixth passage of human hepatitis A virus in FRhK6 cells were diluted 10⁻⁴-10⁻⁹ and 1 ml inoculated onto FRhK6 cell cultures. Coverslips were examined by direct

immunofluorescence through 18 and 20 days, respectively, following inoculation.

Immune adherence (IA) assay. Cell culture harvests at all passages of hepatitis A virus were tested for amount of antigen by the IA procedure described previously (7). Specificity was established in tests with human serum previously established (9) to be seronegative or seropositive for human hepatitis A.

Radioimmunoassay (RIA). The procedure of Purcell *et al.* (13) was used with the exception that polystyrene beads instead of plastic wells were coated with human hepatitis A antibody. Two tenths ml of antigen sample to be assayed were reacted overnight with the beads in Ausria (Abbott) plates at room temperature. After washing, the beads were incubated for 4 hr at 37° with 0.2 ml of I¹²⁵-labeled human hepatitis A antibody. After washing, the cpm of the test sample was determined and P/N values for each test sample were calculated by dividing the cpm of the test sample by the mean cpm value obtained from five negative control specimens.

Immune electron microscopy. Fluids derived from infected cell cultures that were frozen and thawed were clarified by low speed centrifugation, mixed with convalescent human hepatitis A serum, and examined by electron microscopy. The qualitative and quantitative aspects of the procedure are given in reference 4.

S. labiatus marmoset inoculation. Virus harvests from the fifth passage of hepatitis A virus in FRhK6 cells and from uninfected cell passage control cultures were passed to each of two *S. labiatus* marmosets by usual methods (3). The ICD (isocitric dehydrogenase) values and hepatitis A antibody titers (IA) were followed in specimens taken at weekly intervals. The liver from one of the infected marmosets was removed and assayed for presence of hepatitis A antigen.

Results. Propagation of human hepatitis A virus in liver cell explant cultures of adult S. labiatus. Table I summarizes the findings in the studies to propagate human hepatitis A virus serially in *S. labiatus* marmoset liver cell cultures. The inoculum dilution values of initial seed stock were calculated for each passage level based on an approximate 1:10 dilution at each refeed and on similar dilution

TABLE I. CULTIVATION IN SERIAL PASSAGE OF HUMAN HEPATITIS A VIRUS IN MARMOSET LIVER CELL CULTURES.

	Hepatitis A virus content at serial cell culture passage level ^a					
	0 ^b	1	2	3	4	5
Dilution of inoculum	—	1×10^{-9}	1×10^{-20}	1×10^{-31}	1×10^{-39}	1×10^{-47}
Day of harvest	—	23	27	27	20	17
Hepatitis A virus content as estimated by:						
Immunofluorescence ^{c, d, e}	—	4+	4+	4+	4+	4+
Immune adherence titer	<1	<1	<1	<1	<1	<1
Radioimmunoassay (P/N)	3.1	5.7	5.3	4.4	4.9	6.5

^a Serial passages of control cultures (not inoculated with virus) were tested identically with the virus cultures and all were negative.

^b 0 represents the original inoculum; i.e., 0.2 ml of processed liver extract in 2 ml total medium volume.

^c Four plus immunofluorescence indicates that large numbers of hepatocyte-like cells contain large numbers of fluorescent cytoplasmic granules. In general, 4+ indicated that 75–100% of the hepatocyte-like cells fluoresced.

^d At all 5 passages, fluorescence was successfully blocked by pretreatment with convalescent human hepatitis A sera prior to application of the conjugate. Use of preillness sera in the same manner did not block fluorescence.

^e At the fifth passage level the virus was neutralized; i.e., no fluorescent granules developed, by preincubation of the inoculum with convalescent human hepatitis A sera. Preillness sera from the same patients did not neutralize the virus.

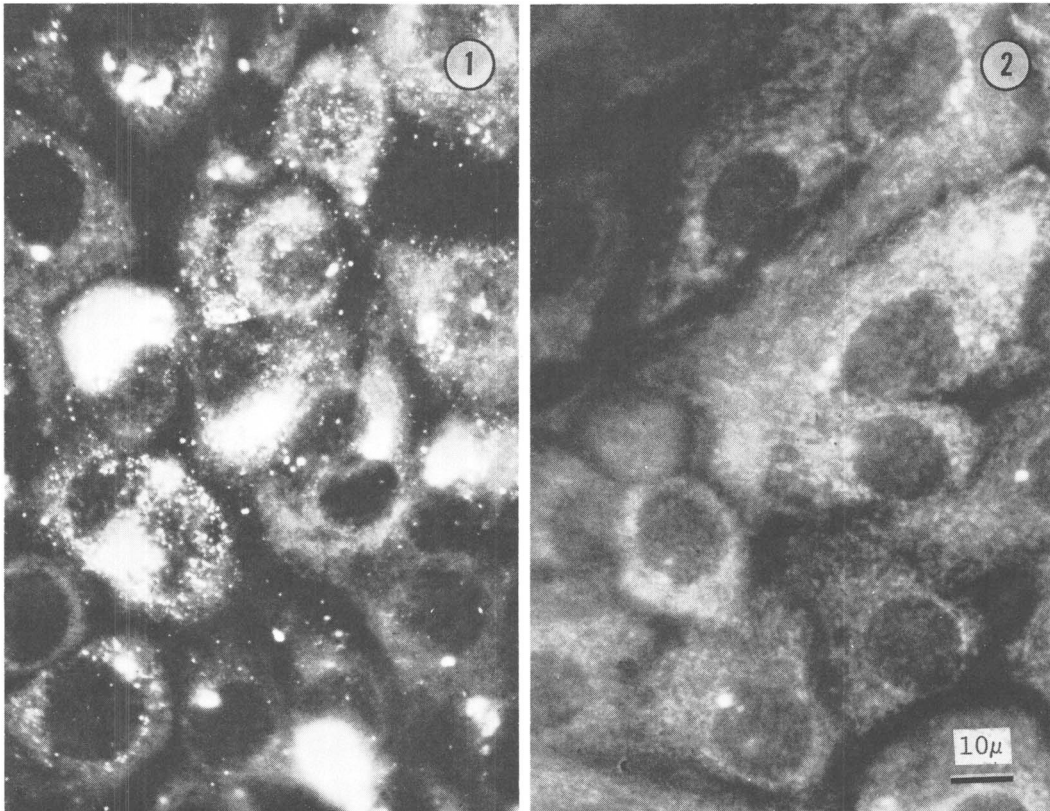


FIG. 1. Photomicrograph of hepatitis A virus-infected hepatocyte-like cells stained by direct immunofluorescence at day 20 of the fourth serial viral passage in liver cells in culture. Fluorescent granules (apple green) are scattered throughout the cell cytoplasm. The larger granules were mostly non-viral and yellow in color.

FIG. 2. Photomicrograph of normal hepatocyte-like cells stained by direct immunofluorescence at day 20 of the fourth serial passage control culture of liver cells. The few large granules that were present were normal cell constituents, staining yellow.

at each serial transfer. No account was taken of the effect of possible adsorption and elution of the virus from cells since there is no way of measuring this possibility. Minute cytoplasmic granules with apple-green fluorescence were detectable in some of the hepatocyte-like cells as early as 8 days after inoculation in the initial cell culture passage. Both the numbers of affected hepatocyte-like cells and the numbers of immunofluorescent granules per cell increased with time so that by the time of harvest on day 23, 75–100% of hepatocyte-like cells but not other cell types contained large numbers of immunofluorescent granules. No cytopathic changes were seen and no specific fluorescence was observed in uninoculated control cultures. Fluorescence was blocked in this and all subsequent passages by convalescent, but not the preillness, human hepatitis A sera as measured in the immunofluorescence blocking assay. Figures 1 and 2 show the typical appearance of hepatitis A virus-infected hepatocyte-like cells and uninfected control cells that were stained by direct immunofluorescence. Infectivity of the virus obtained at fourth passage was specifically neutralized by human hepatitis A convalescent, but not by

preillness, sera. Hepatitis A antigen was not detectable in cell culture harvests by IA assay but was detected by the more sensitive radioimmunoassay. An adventitious viral agent was detected by electron microscopy at passage 5 in both the inoculated and uninoculated cell cultures. This was not detected at earlier passages and appeared to have come from the liver cell culture that was used for passage 5. Because reliable propagation of hepatitis A virus had been established by passage 5 and because successful passage in FRhK6 cells was also accomplished, no further passages were made in *S. labiatus* liver cultures.

Propagation of human hepatitis A virus in FRhK6 cell cultures. Marmosets are of limited availability and alternate cell culture substrates were sought. Early studies here revealed successful propagation of hepatitis A virus in primary cell culture of kidneys of *Cercopithecus aethiops* monkeys but many of the cultures, when incubated for 3 or 4 weeks, were shown to have present cytopathic adventitious agents of endogenous origin. Consequently, a serially propagated cell was sought. Table II shows the findings in studies of the propagation of the human hepatitis A

TABLE II. CULTIVATION IN SERIAL PASSAGE OF HUMAN HEPATITIS A VIRUS IN FRhK6 CELL CULTURES.

	Hepatitis A virus content at serial cell culture passage level ^a								
	0 ^b	1	2	3	4	5	6	7	8
Dilution of inoculum	—	1×10^{-6}	1.6×10^{-9}	2.6×10^{-12}	4.2×10^{-15}	6.7×10^{-18}	1.1×10^{-20}	1.8×10^{-23}	2.9×10^{-26}
Day of harvest	—	23	11	12	12	12	10	11	9
Hepatitis A virus content as estimated by:									
Immunofluorescence ^{c,d,e}	—	4+	4+	4+	4+	4+	4+	4+	4+
Immune adherence	<1	2	1	1	1	2	4	2	4
Radioimmunoassay (P/N)	3.1	31.6	10.9	11.3	15.5	22.4	46.5	23.2	25.8
Titration in FRhK6	N.D. ^f	N.D.	N.D.	N.D.	N.D.	10 ⁶ (18)	10 ⁷ (20)	N.D.	N.D.
Marmoset inoculation ^g	Infectious	N.D.	N.D.	N.D.	N.D.	infectious	N.D.	N.D.	N.D.

^a Serial passages of control cultures (not inoculated with virus) were tested identically with the hepatitis A virus cultures and all were negative.

^b The zero inoculum consisted of 0.2 ml of processed liver extract in 2 ml vol of culture medium.

^c Four plus (4+) immunofluorescence indicates that the entire FRhK6 cell sheet contained large numbers of fine fluorescent cytoplasmic granules.

^d At all 8 passages immunofluorescence was successfully blocked by pretreatment with convalescent human sera from hepatitis A patients prior to application of the conjugate. Pre-illness sera used in the same manner did not block fluorescence.

^e Virus harvested from the fifth passage was successfully neutralized; i.e., no fluorescent granules developed through 21 days of incubation by treatment of the inoculum with convalescent human hepatitis A sera. Preillness sera from the same patients did not neutralize the virus.

^f N.D. = not done. Figures in parentheses are numbers of days of inoculation.

^g The liver extract used in the original inoculum was infectious for marmosets as explained in Materials and Methods. Most importantly, the harvest of virus passage 5 in cell culture, diluted 1:10 in PBS, induced hepatitis A disease in two of two marmosets injected with 1 ml iv. One animal showed serum enzyme elevation at day 7, the other at day 21. Both developed hepatitis A virus antibody. Liver was removed from one animal and showed hepatitis A virus antigen detectable by radioimmunoassay. Cell culture passage controls did not induce hepatitis.

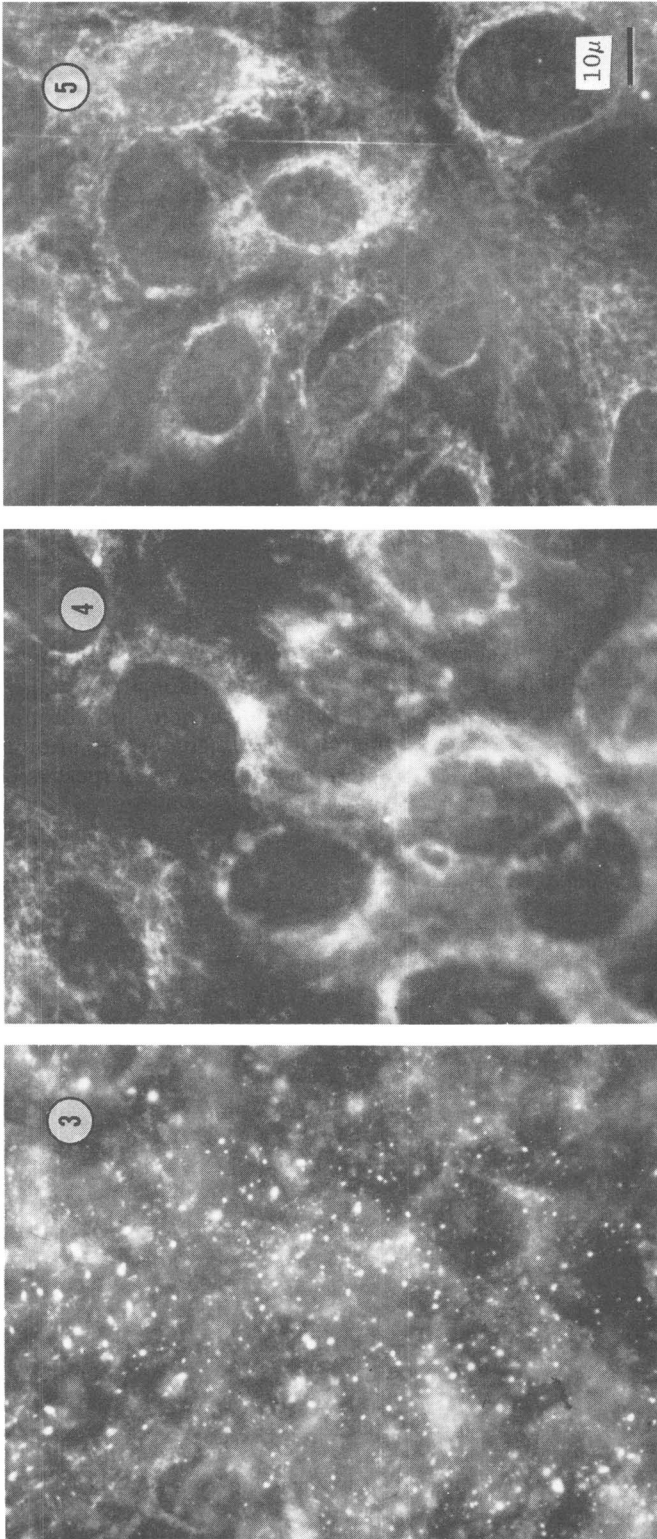


FIG. 3. Photomicrograph of hepatitis A virus-infected FRhK6 cells stained by direct immunofluorescence at day 12 of the fifth serial viral passage in FRhK6 cells. Cell sheet pretreated with preillness serum of pt. 109-06. Numerous fluorescent granules (apple green) are evident.

FIG. 4. Photomicrograph as in Fig. 3, but cell sheet was pretreated with convalescent serum of pt. 109-06. No fluorescent granules were present.

FIG. 5. Photomicrograph of normal FRhK6 cells at day 12 of the fifth serial passage control culture, stained by direct immunofluorescence. No fluorescent granules were present.

virus in FRhK6 cell cultures. Small numbers of minute cytoplasmic immunofluorescent granules were seen as early as 3 days after inoculation with hepatitis A virus. Extensive development of apple-green immunofluorescent granules occurred throughout the cell sheets during the following weeks. The incubation period for development of immunofluorescence was shortened on serial passage, but there was no evident cytopathic effect. The virus was serially propagated *in vitro* and sequential dilution of the original inoculum was as shown in the table. Viral immunofluorescence was specifically blocked by human hepatitis A convalescent serum at all passage levels. Figures 3, 4, and 5 show, respectively, immunofluorescence of cells inoculated with virus in fifth passage and pretreated with preillness human hepatitis A serum, of cells inoculated with the same virus but pretreated with convalescent human hepatitis A serum, and of uninoculated control cells. The virus in the fifth passage harvest was specifically neutralized by human convalescent hepatitis A sera but not by preillness sera.

The human hepatitis A virus was propagated with larger yield in the FRhK6 cells than in the liver cell cultures, probably because of the larger proportion of susceptible cells in the fetal kidney cell cultures. As seen in Table II, hepatitis A antigen was detectable in FRhK6 cells by IA assay as well as by radioimmunoassay. Titration of the infectivity of the virus harvests of the fifth and sixth passages in the FRhK6 cells gave titers of about 10^6 and 10^7 infectious units per ml, respectively. Virus in the fifth passage harvest retained the ability to infect *S. labiatus* marmosets.

The findings in further tests to establish the identity of the agent propagated in FRhK6 cells as human hepatitis A virus are given in Table III. The virus, in fifth passage harvest, reacted specifically and identically with marmoset-propagated virus in IA tests of sera from human cases of hepatitis A in which the antibody was present. Further to this, virus present in FRhK6 cells at the fourth passage level was morphologically identical to human hepatitis A virus as previously described (4, 14). Figure 6 shows an electron microscopic picture of 27 nm particles present in the cell culture fluid, coated and aggregated by hu-

TABLE III. IMMUNE ADHERENCE ASSAY FOR HEPATITIS A ANTIBODY IN HUMAN SERA TESTED USING INFECTED MARMOSET LIVER-DERIVED AND FRhK6 CELL CULTURE-DERIVED ANTIGENS.

Subjects	Case Nos.	Time of specimen (Days)	Antibody titer	
			Liver extract ^a Anti-gen	Cell culture Anti-gen ^b
Hepatitis A cases	033-02	-21	<5	<5
		+36	≥2560	≥2560
		+102	≥2560	≥2560
	056-08	-57	<5	<5
		+185	≥2560	≥2560
		34309	-6	<5
Hepatitis B cases	039-01	+3	160	160
		+104	≥2560	1280
		-108	<5	<5
	03903	+10	<5	<5
		+149	<5	<5
		-27	≥2560	1280
Normal human sera	2	+7	≥2560	≥2560
	16	+192	≥2560	1280
	20		640	160
			<5	<5
			<5	<5

^a Tests were performed in March 1976 using a standard CsCl-purified hepatitis A viral antigen derived from *in vivo* infected rufiventer marmoset liver (9).

^b Cell culture antigen was the harvest from the fifth serial passage of hepatitis A virus *in vitro* in FRhK6 cell culture.

man hepatitis A antibody.

Discussion. The present report describes the first reliable propagation of human hepatitis A virus in cell culture. Though propagation of human hepatitis A virus has been claimed by several workers during the past several decades, these purported agents were found to be spurious isolates and not the virus of hepatitis A (1, 15-22).

The present studies, stemming from propagation of the virus in marmoset monkeys, have shown definitive proof for serial propagation of the virus in cell cultures both of primary explanted marmoset liver and in serially propagated fetal rhesus monkey kidney cells, FRhK6.

Viral propagation in marmoset liver cultures appeared limited to cells resembling hepatocytes and the virus was not detected in other cell types. The sequence of 5 passages with resultant dilution of original inoculum to a theoretical 10^{-47} dilution precludes any possibility for carry-over of virus from the original inoculum. Replication in cell culture

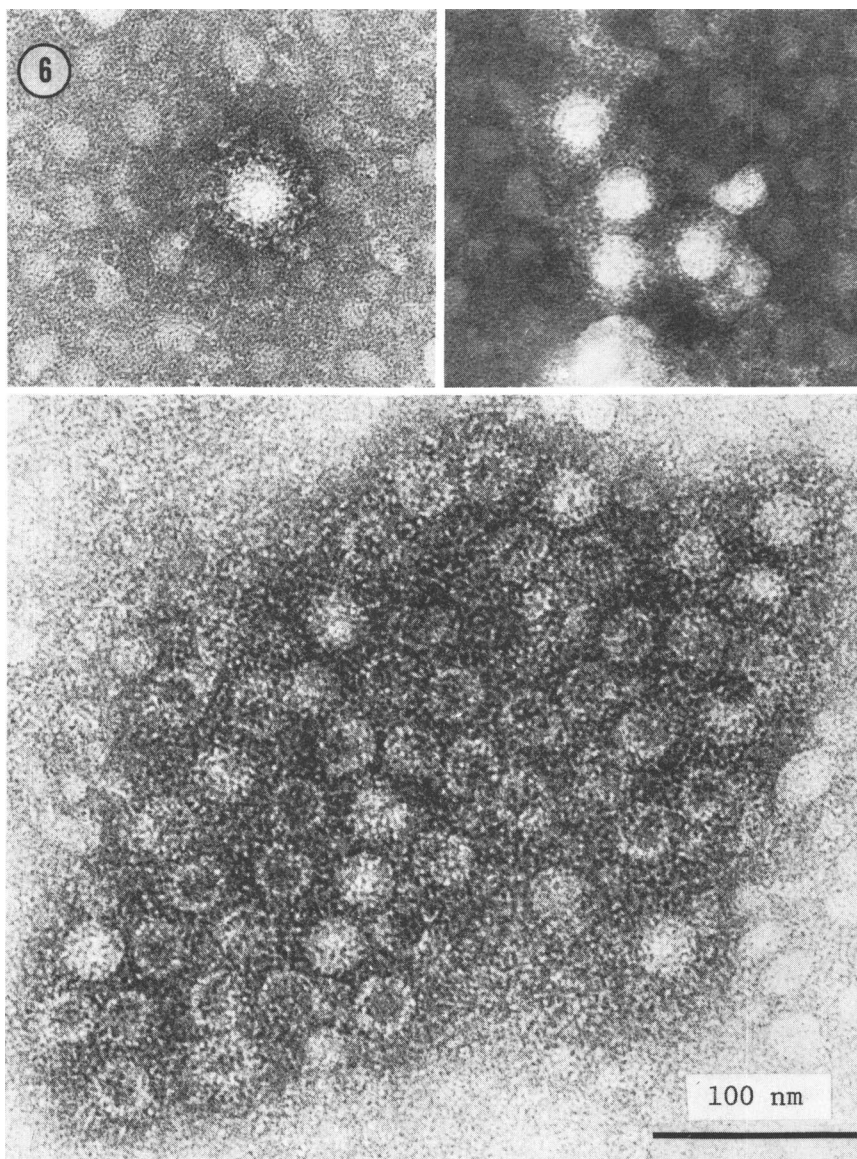


FIG. 6. Electron micrograph of hepatitis A virus particles aggregated with antiserum as detected in the fourth serial passage of the virus in FRhK6 cells. Phosphotungstic acid stained.

was supported further by the progressive development in cell cultures of antigen detected by immunofluorescence and by the demonstration of presence of antigen detectable by the radioimmunoassay procedure. Blocking immunofluorescence assays and serum neutralization tests with paired sera from human cases of hepatitis A infection established the serologic identity of the virus as human hepatitis A.

Quantitatively greater replication of virus

was obtained in the serially passaged normal fetal rhesus kidney cell line (FRhK6). Essentially all cells in these cultures appeared to replicate the virus. The virus was successfully transferred through 8 serial passages in which the calculated dilution of original inoculum was 2.9×10^{-26} . The quantity of antigen was far greater than in liver cell cultures, permitting detection of antigen by IA assay and achieving infectivity titers as high as 10^7 infectious units/ml at passage 6. Identity of the

virus as hepatitis A was established in blocking immunofluorescence and in serum neutralization tests with paired sera from human hepatitis A cases as described above. Additionally, identity was established in IA assays of paired sera from human hepatitis A cases, in immunologic and morphologic identification by immune electron microscopy, and in hepatitis induction in *S. labiatus* marmosets.

There are three major reasons for development of a means for propagation of human hepatitis A virus *in vitro*. One is to provide a means for detecting human hepatitis A virus; another is to provide a means for preparing virus and antigen for use in serologic assays, and the third is to provide a source of virus or antigen to prepare vaccines. The investigations of the recent time period have shown that all three objectives have been met by use of marmosets. The marmoset, however, is in limited supply and, in fact, is on the list of endangered species. The findings in the present study do not at present provide the means for substituting cell culture for animal propagation of the virus, primarily because marmoset liver cell culture does require the use of marmosets and because the FRhK6 cell is capable only of limited serial propagation, viz., 12 passages at most (11). Hepatitis A virus has, unfortunately, proved noncytopathic in these cell cultures to date. Propagation in adult monkey kidney, while reliable, is fraught with the problem of indigenous viruses that replicate simultaneously and negate the meaningfulness of investigations. The findings in the present studies do, however, point the way to production of virus in some cell that can be propagated en masse. Human diploid lung fibroblast cells, commonly used to prepare virus for vaccines, have been found to give only limited replication of hepatitis A virus to date (unpublished findings).

Summary. Human hepatitis A virus was reliably and repeatedly propagated in primary explant cell cultures of marmoset livers and in the normal fetal rhesus kidney cell line (FRhK6). Identity of virus was established in immunofluorescence, immunofluorescence blockade, serum neutralization, immune adherence, radioimmunoassay, immune electron microscopy, and marmoset inoculation tests. The virus propagated to greatest extent

in FRhK6 cells. No cytopathology was observed. These studies represent the first reliable propagation of human hepatitis A virus *in vitro* and point the way to the eventual means for detection and quantification of live virus *in vitro* and of production in cell culture of virus for diagnostic antigen and vaccine preparation.

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