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PROPAGATION OF HEPATITIS A VIRUS IN A RENAL CELL LINE JTC-12.P3 OF CYNOMOLGUS MONKEY ORIGIN

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Summary. – Human hepatitis A virus (HAV) derived from 10 % HAV infected marmoset liver homogenate and faeces from acute hepatitis A was successfully propagated *in vitro* in a new cell line, JTC-12.P3. The cell line originated from the renal cortex of cynomolgus monkey which was adapted to growth in a serum free, protein free, chemically defined synthetic medium. Replication of the virus was followed by solid phase RIA, immunofluorescent staining, and immunoelectron microscopy. The propagation of HAV occurred over several passages, with the 1st and 2nd passages requiring at least 8 weeks each. However, with the increasing serial passage of virus, the period needed to detect it was shortened, suggesting the adaptation of HAV to the cells. The identity of the newly synthetized virus particles with HAV was established by immunoelectron microscopy and immunofluorescent blocking effect with human convalescent serum. The HAV propagated in JTC-12.P3 cells banded predominantly at a density of 1.32 g/cm^3 in CsCl gradient. The infected cells showed no specific signs of CPE. Ultrastructurally, clusters of virus particles 27 nm in diameter were observed mainly in the lysosomal vesicles and freely in crystalline array in the cytoplasm, too. Addition of 0.1 % of various anti-HAV negative sera or of prostaglandin E_1 to the culture medium caused accelerated propagation of HAV.

Key words: hepatitis A virus; JTC-12.P3 cell line; propagation

Introduction

Since the electron microscopic visualization of HAV and successful transmission of type A hepatitis into the marmoset in 1973 (Feinstone *et al.*, 1973), a new era of HAV study has been opened. Then Provost and Hilleman (1979) reported the propagation of HAV *in vitro* in cultured cells. Thereafter, several reports followed, describing the successful propagation of HAV *in vitro* and further virological studies. Then HAV has been classified as an enterovirus. However, some different features of the biological behaviour of HAV from those of other

enteroviruses have been noted, especially with *in vitro* propagation. Unlike most other enteroviruses, HAV causes no obvious CPE in cell cultures. Moreover, the primary culture period for propagation needs 8 to 10 weeks until the appearance of HAV antigen (HAVAg) in the cells. However, an adaptation with more rapid propagation occurs in subsequent passages. These facts indicate some discrepancies with the clinical features of type A hepatitis which *in vivo* showed abundant faecal excretion of HAV and acute hepatic injury after a rather short incubation period. To clarify the cause of this discrepancy we attempted to propagate HAV in a new cell line derived from the cortex of the kidney of cynomolgus monkey, designated JTC-12.P3, which was grown in a chemically defined synthetic medium. This report describes the adaptation and efficient propagation of HAV in the JTC-12.P3 cell line and some aspects concerning HAV growth.

Materials and Methods

Virus. The NF-9 and K-5 stool strains of HAV (Shibayama *et al.*, 1985) were used.

Cell line and culture conditions. The JTC-12.P3 cell line used was established from the foetal cynomolgus monkey kidney and has been maintained in a serum-free chemically defined synthetic medium, DM-160 (Kyokuto Co. Ltd., Tokyo, Japan) since 1969 (Takaoka *et al.*, 1962; Takaoka and Katsuta, 1971). Cells were cultured at 37 °C in slanted stationary culture bottles (15 × 150 mm) and the medium was renewed twice a week.

Virus inoculation. When JTC-12.P3 cell line reached confluence, the medium was removed and 0.1 ml of virus suspension (NF-9 or K-5 stool) was added to the cells for 1 hr at 37 °C. Then 2 ml of medium was added to continue with renewal of the medium twice a week. From the 2nd passage onwards, 0.2 ml of HAV-infected cell extract was inoculated into the culture.

Harvesting of cell-associated HAV extract. At selected intervals after virus inoculation, medium was removed from the culture bottles and cell sheets were washed 2–3 times with Ca^{++}- and Mg^{++}-free PBS (PBS(–)). Cells with 2 ml of PBS (–) were frozen and thawed 2–3 times and then centrifuged at 3000 rpm for 15 min. The supernatant thus obtained was used as the cell-associated HAV extract. Specimens of this extract and the culture medium were stored at –40 °C until use.

RIA for HAVAg. HAVAg was detected using an HAVAB-M kit (Abott Laboratories, North Chicago) with slight modification as described previously (Kojima *et al.*, 1981). The ratio of the sample cpm to that of the negative control was determined, and a ratio greater than 2.1 was considered positive.

Immunofluorescent microscopy. HAV-infected JTC-12.P3 cells were cultured on coverslips, fixed with cold acetone for 7 min and washed with PBS(–). After incubation with anti-HAV-positive human serum for 12 hr at 4 °C and washing 2–3 times with PBS(–), cultures were incubated with FITC-conjugated goat anti-human IgG for 12 hr at 4 °C. The cultures were again washed with PBS(–) and observed under a fluorescent microscope.

Electron and immunoelectron microscopy by immunocolloidal gold method were performed as described previously (Miyamoto *et al.*, 1971). Protein A colloidal gold particles (5 nm, 6μg/ml) or goat anti-human IgG colloidal gold particles (5 nm, 70μg/ml) (E-Y Laboratories, Inc., San Mateo, CA) were used to detect the anti-HAV antibodies.

Isopycnic banding in cesium chloride gradient. A 0.5-ml aliquot of each supernatant was layered onto an 11 ml continuous cesium chloride gradient (1.3 – 1.5 g/cm^3) prepared in 50 mmol/l Tris buffer pH 7.4 containing 100 mmol/l NaCl and 0.1 % N-lauroyl sarcosine (TNS buffer). After centrifugation at 200 000 x g for 18 hr, 18 fractions were collected from each tube. The density of each fraction was determined by refractometry and the presence of HAVAg was tested by RIA as described above.

Table 1. Cell-associated HAV antigen after each passage. Sample/negative control ratio (S/N)

1st passage

	1st culture period (weeks)				
	4	8	12	16	20
	21.9	18.3	25.8	31.7	34.3

2nd passage

1st culture period (weeks)	2nd culture period (weeks)			
	4	8	12	16
4	(-)	(-)	N.D.	N.D.
8	(-)	N.D.	3.1	16.5
12	(-)	3.3	N.D.	N.D.
16	6.3	43.2	80.8	N.D.

3rd passage

Culture period (weeks)		3rd culture period (weeks)			
1st	2nd	4	8	12	16
4	4	(-)	N.D.	(-)	(-)
	8	(-)	(-)	N.D.	N.D.
8	4	(-)	(-)	(-)	N.D.
	12	(-)	6.7	37.6	N.D.
	16	(-)	33.9	157.5	N.D.
12	4	(-)	(-)	(-)	N.D.
	8	(-)	(-)	46.9	N.D.
16	4	(-)	65.6	152.2	N.D.
	8	7.6	104.9	162.8	N.D.
	12	54.4	135.5	235.2	N.D.

(-) denotes S/N < 2.1; N.D. = not done.

Specificity of the propagated HAV. To investigate the specificity of the HAV obtained from infected JTC-12.P3 cells, cell extract from the 6th passage was used to detect anti-HAV IgM antibody in serum from patients with acute hepatitis A, B, non-A non-B and healthy controls.

Effect of sera or hormones on HAV propagation. After the inoculation of 6th passage material, culture was done with or without 0.1 % anti-HAV-negative human placental or, adult human serum as well as with foetal bovine serum and calf serum. Hormone supplementation was carried out in the same way using insulin, predonisolone and prostaglandin E_1 at a final concentration of 10^{-6} mol/l.

At 1, 2 and 4 weeks after inoculation, cell-associated virus was detected by RIA as described above.

Results

Propagation of HAV in JTC-12.P3 cells

In the 1st passage, 0.1 ml of NF-9 strain was inoculated. Detection of HAVAg in cells after inoculation was performed every 4 weeks. Table 1 and Fig. 1 show S/N ratios of cell-associated HAVAg at various times after inoculation. The values of S/N ratios slowly increased with time. In the following passages, cells were inoculated with 0.2 ml of the specimens for cell-associated virus of prior passage, and cultured as described earlier.

The 2nd and following passages, started with an inoculation of a 4 weeks specimen of the 1st passage, did not produce detectable amounts of HAVAg even after 24 weeks of subsequent passages. However, specimens started with

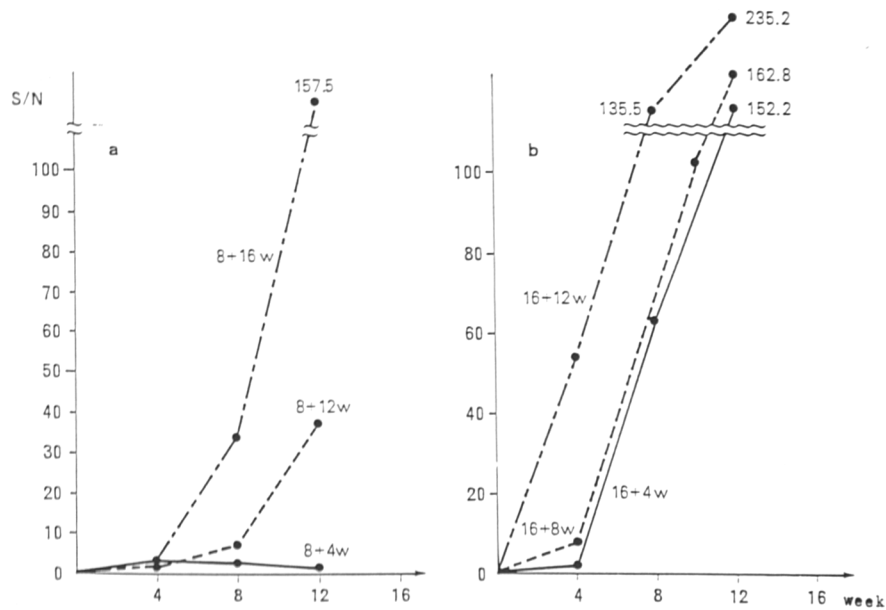

Fig. 1
Propagation of HAV: cell-associated antigen of the 3rd passage

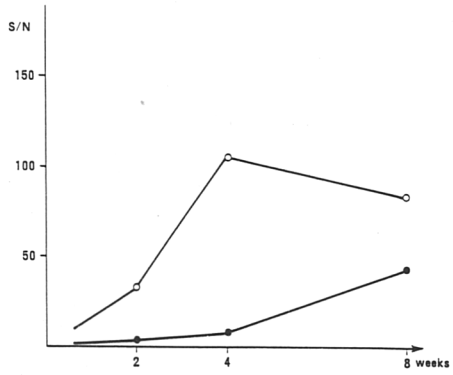

Fig. 2
Propagation of HAV: cell-associated antigen of the 2nd and 6th passages
●——● 2nd passage, S/N 24.9; ○——○ 6th passage, S/N 22.3.

the inoculation of 8, 12 and 16 weeks specimens of the 1st passage showed positive results after 4 or more weeks in the subsequent passages. E.g., samples given 8 weeks of the 1st passage and 12 or 16 weeks of the 2nd passage (8 + 12 W, or 8+ 16 W) gave positive S/N ratios of 3.1 or 16.5, respectively. In 2nd or 3rd passages, however, rather high S/N ratios were obtained in specimens of 16+ 12 W, 8+ 12+ 12 W, or 8+ 16+ 8 W. In other words, at least 8 weeks cultivation in the 1st passage seemed to be necessary to obtain positive results in subsequent passages, and a total of around 30 weeks of cultivation in the 1st two or three passages seemed to be necessary to obtain rather high S/N ratios. When the

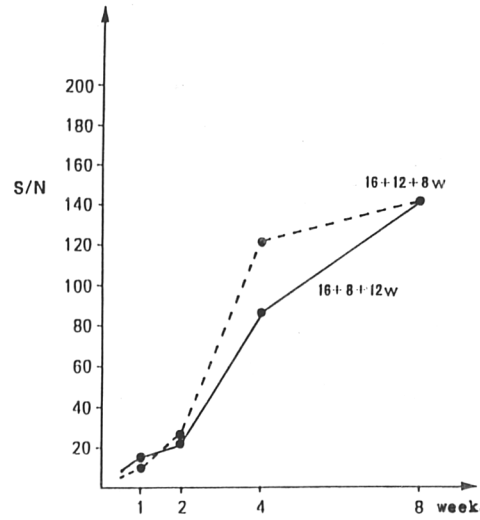

Fig. 3
Propagation of HAV: cell-associated antigen of the 4th passage

antigen titer of the HAV extract, taken from the 2nd and the 6th passages, was adjusted to fixed S/N ratio, the increase in HAV after inoculation in the case of the 6th passage was detected much earlier and was thought to indicate adaptation of HAV to the cells (Fig. 2). Afterwards, as shown in Fig. 3, HAV in the 4th passage could be detected in an early period of cultivation, being already positive at 1 week. At 4 weeks or more, plateau-type high values were shown. Throughout the entire period of observation, no CPE was obtained even in higher passages with high titers of HAVAg.

Detection of HAVAg in supernatant fluid

Fig. 4 shows the appearance of HAVAg in the supernatant of infected cells. In the 1st and 2nd passages, there was no detectable amount of HAVAg in the supernatant (Fig. 4*a*, *b*). However, in the 3rd passage, small amounts of HAVAg were detectable in the supernatant as cell-associated virus, increasing 8 weeks after inoculation. In the 7th passage, moderate amounts of HAVAg could be detected in the supernatant (Fig. 4*c*, *d*).

In the experiment with inoculation of K-5 stool strain of HAV to cells, the successive virus propagation over several passages was confirmed, too (data not shown).

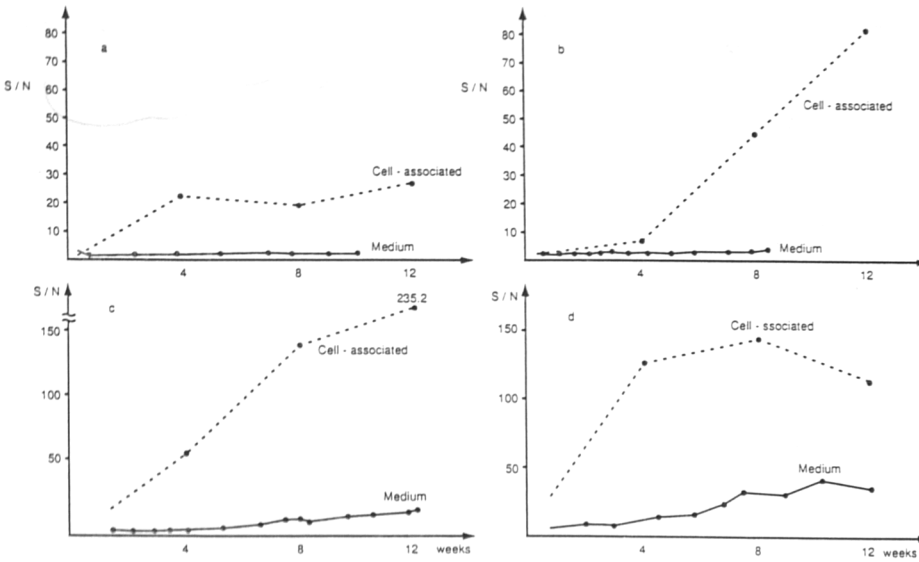

Fig. 4
Detection of HAV in medium and cells in culture
1st passage (a), 2nd passage (b), 3rd passage (c), 7th passage (d).

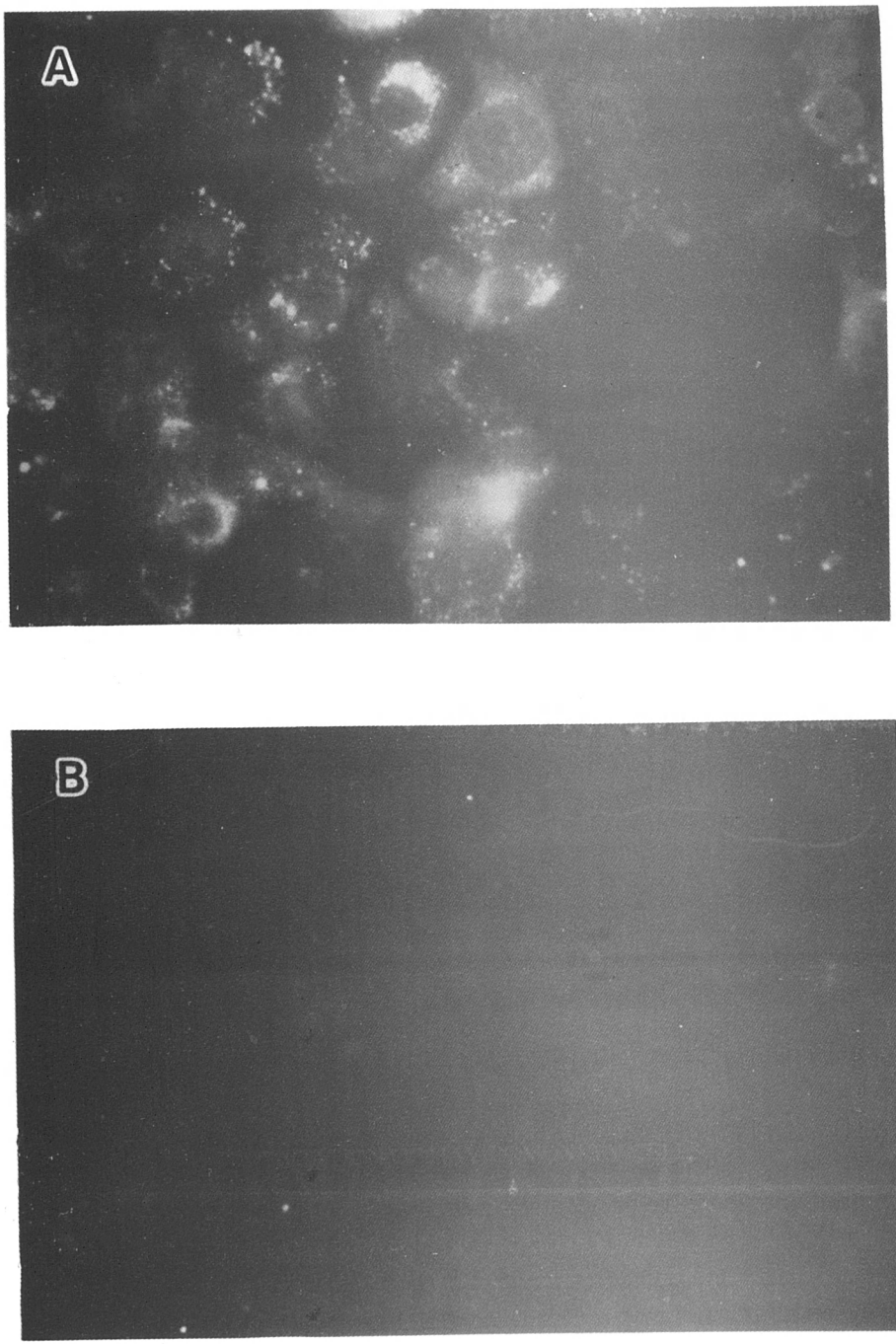

Fig. 5
For legend see page 216.

Table 2. Detection of IgM class anti-HAV antibody in patients' sera using in vitro propagated HAV in place of standard HAVAg in HAVAB-M kit

Cases	Patient	HAVAg of this study*	Standard HAVAg
Acute hepatitis A	A.M.	(+)	(+)
	M.K.	(+)	(+)
Acute hepatitis B	Y.B.	(-)	(-)
	S.I.	(-)	(-)
Acute hepatitis non-A non-B	M.S.	(-)	(-)
	H.N.	(-)	(-)
Normal (HAV non-infected)	M.T.	(-)	(-)
	H.H.	(-)	(-)

*Cell-associated HAV from the 5th serial passage in JTC-12.P3 cells.

Immunofluorescent microscopy

In Fig. 5 the localization of intracellular HAV in the 3rd passage by indirect immunofluorescence method is shown. The characteristic, specific granular fluorescence of HAV is seen in most of the cells, within the cytoplasm, and especially in the perinuclear area.

Electron microscopic study

Cells infected with HAV for 4 weeks displayed proliferated vacuoles, lysosomes, and many amorphous matrix materials in the cytoplasm. Virus particles of 20–27 nm in diameter were seen mainly in lysosomal vesicles and freely in the cytoplasm or in crystalline array associated with the viral matrix (Fig. 6). The specificity of the HAV particles was confirmed by indirect immunocolloidal gold staining.

Isopycnic banding

Fig. 7 shows the isopycnic banding of HAV extracted from infected cells. The main peak appeared at a density of 1.32 g/cm^3.

Specificity of HAV propagated in JTC12.P3 cells

Concerning the specificity of the HAV, while it was confirmed by the RIA method that a 100 % blocking effect was attained by preincubation with anti-HAV antibody-positive or negative human sera, when the standard HAV of the HAVAB-M kit for measurement of IgM-class anti-HAV antibody was replaced

Fig. 5

Photomicrographs of HAV-infected cells stained by indirect immunofluorescence
Infected cells, 3rd passage at 4 weeks (A), uninfected cells (B). (Magn. × 1350.)

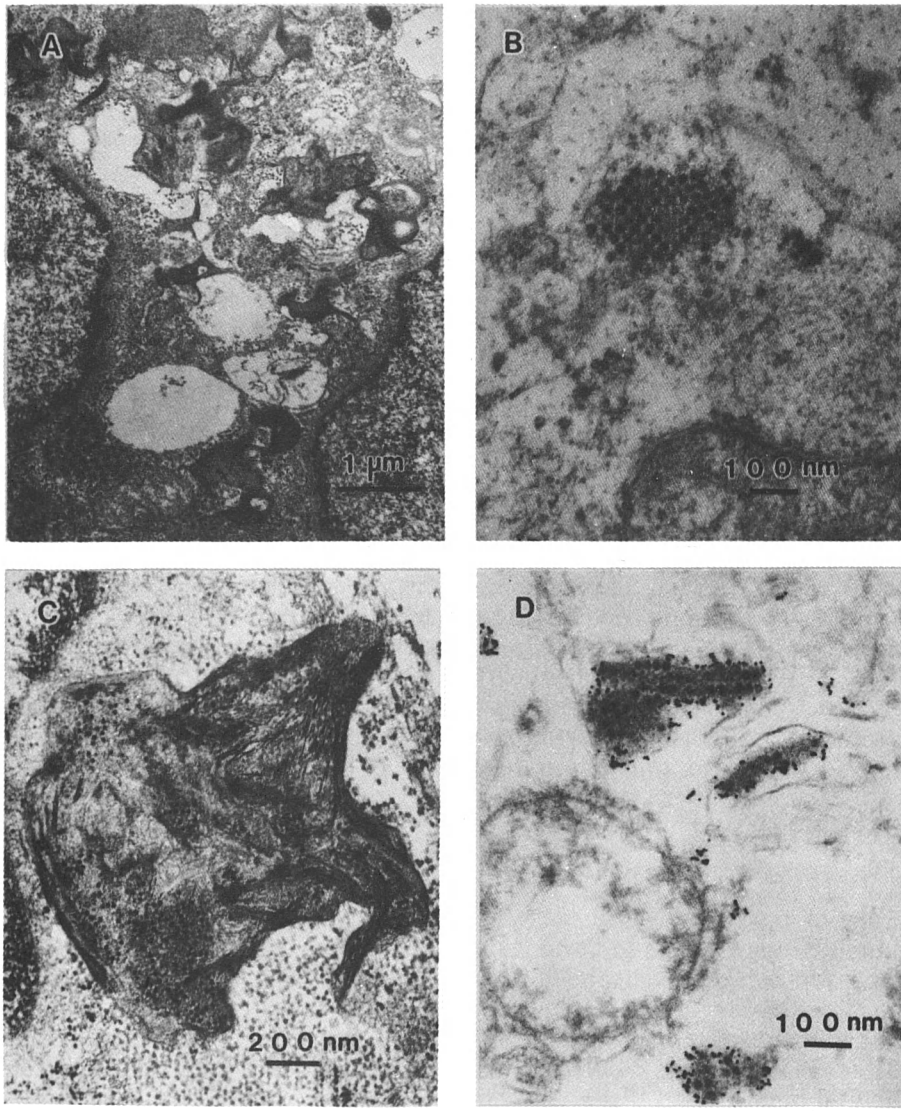

Fig. 6
Electron micrographs of HAV-infected cells
Infected cell (A, magn. × 17 300); crystalline array of virus particles in cytoplasm (B, magn. × 100 000); virus particles in amorphous matrix (C, magn. × 53 000); virus particles stained indirectly by immunocolloidal gold (D, magn. × 100 000).,

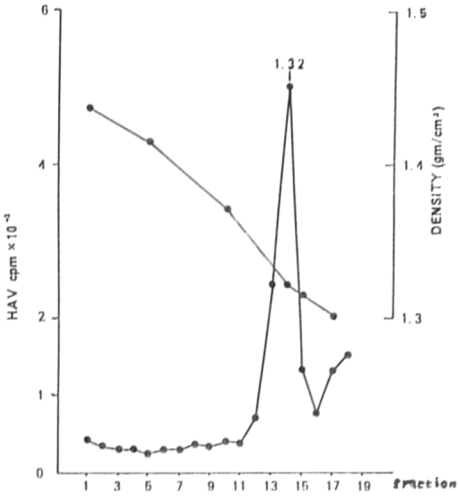

Fig. 7
HAVAg detected by RIA in fractions from CsCl gradient

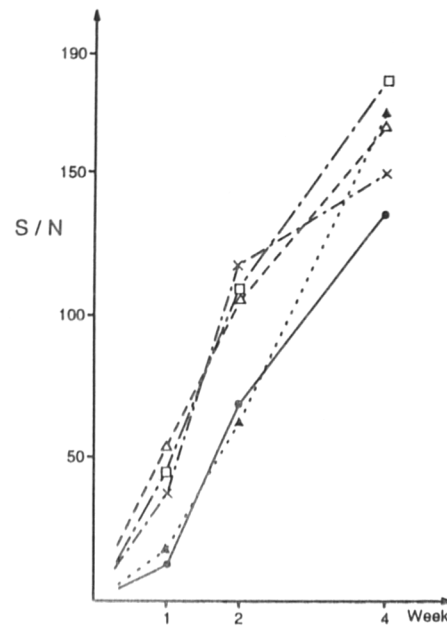

Fig. 8
Effect of serum on propagation of HAV in cell culture
Cell-associated antigen assayed. ●——● no serum; △- - - -△ anti-HAV antibody-negative human placental serum; x-·-x anti-HAV antibody-negative human adult serum; □——□ foetal bovine serum; ▲- - - -▲ calf serum.

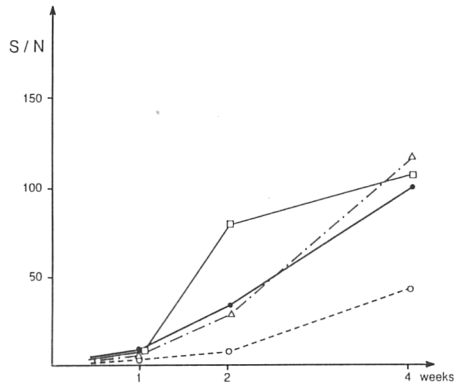

Fig. 9
Effect of hormones on propagation of HAV in cell culture
Cell-associated antigen of the 5th passage assayed. ●——● no hormone; ○- - - -○ insulin; △— · —△ predonisolone; □——□ prostaglandin.

by an HAV extracted from infected cells, measurement of HAV-IgM antibody in cases of hepatitis A, B, non-A, non-B and in normal subjects revealed that only in hepatitis A was the result 100 % positive (Table 2). These results confirmed the specificity and usefulness of our HAV strain.

Effects of sera or hormones on the propagation of HAV in JTC-12.P3 cells

As shown in Fig. 8, human placental, adult human, and foetal calf serum supplementation enhanced the production of HAVAg in this system at 2 weeks after inoculation compared with no serum supplement. However, at 4 weeks after inoculation there were no marked differences.

Fig. 9 shows the results of the effect of hormone supplementation. At 2 weeks after inoculation prostaglandin E_1 enhanced production of HAVAg, while insulin suppresed it.

Discussion

The propagation of HAV *in vitro* was first reported by Provost and Hilleman (1979) in an experiment using primary cultured liver cells of the marmoset and Frhk-6 cells.

Since then, the propagation of HAV has been confirmed in a variety of cells such as primate cells (Frhk-4/R, AGMK, BSC-1), MRC-5 and PLC/RPF/5 cells (Flehmig, 1980; Daemer *et al.*, 1981; Deinhardt *et al.*, 1981; Flehmig, 1981; Flehmig *et al.*, 1981; Gauss-Müller *et al.*, 1981; Kojima *et al.*, 1981; Binn *et al.*, 1984; Friedman-Alvermann *et al.*, 1985; Quan *et al.*, 1986; Ashida *et al.*, 1989). The findings observed commonly in those experiments are as follows: (1) In the culturing of the 1st viral passage, the period from the HAV inoculation until the appearance of HAVAg, or that of the 2nd passage, needed at least 8–10 weeks. (2) With the serial passage of the virus *in vitro*, the period until the detection of

HAVAg decreased. (3) Even after serial passages, the infection became persistent without CPE in the cell culture. Concerning HAVAg found in the media, findings ranged from no HAVAg determined in some reports to abundant discharge seen in other reports. These *in vitro* phenomena of HAV are considerably contrary to the *in vivo* effect in the liver which is cytopathic, and is immediately detectable as faecal excretion of the virus in patients contracting hepatitis A. Therefore, it is considered that host immune response rather than CPE of the virus itself may be involved in the liver injury by HAV (Kurane *et al.*, 1985; Vallbracht *et al.*, 1986). In the present study, for the purpose of clarifying the apparent contradiction, we attempted to propagate HAV in a new cell line of a primate origin, namely JTC-12.P3 cells derived from the kidney of the cynomolgus monkey (Takaoka *et al.*, 1962, 1971), and upon successful propagation we examined the resulting HAV. These cells were cultured in a serum- and protein-free chemically defined synthetic media. Derived from the cortex of the kidney, these cells are sensitive to parathyroid hormone and prostaglandin E_1 and have specific cellular functions of its kidney origin (Ishizuka *et al.*, 1978). HAV strain (NF-9) passaged in marmosets and a wild strain of HAV isolated from the stool of a patient with hepatitis A (K-5) were inoculated into cell cultures, where favorable virus propagation was confirmed. At 4 and 8 weeks of the 1st passage after HAV inoculation, the S/N ratios of cell-associated HAVAg were relatively high (21.9 and 18.3). However, in the case of inoculation from a specimen of the 1st passage at the 4th week, which was cultured for over 20 weeks in the following passage, no HAVAg appeared. Therefore it is considered that HAV propagation in cells at the 4th week of the 1st passage was incomplete and limited. After the 3rd passage, the period needed for HAV appearance became shorter after each successive passage. Finally, the expression of antigen became so highly positive that about 10 % of intracellular HAVAg was discharged into the media. However, even for this cell line, the 1st passage took over 8 weeks to develop high level of virus propagation. Therefore, it was considered that a total of 30 weeks was necessary for completion of the 1st, 2nd and 3rd passages of HAV in these cells to finally attain rapid propagation. However, in the 9th passage, HAVAg appeared within a few days after inoculation. The discharge into the media was observed when intracellular HAV propagation reached certain levels. It is this so-called discharge of mature virus, which should be further studied. Moreover, HAV propagation was studied in the cell line after serum and hormone supplementationn which affected the propagation and function of the cells. The serum and hormone which enhanced the propagation and function of the cells simultaneously facilitated the propagation of HAV. CPE was not observed in the cells even after over 10 viral passages, suggesting a persistent infection.

As the present experiments, also most previous reports on *in vitro* propagation of HAV have not indicated any occurrence of CPE. Recently, however, it has been reported that CPE was observed after repeated viral passages (Cromeans *et al.*, 1989). Thus, it is likely that CPE might initiate after a sufficient number of

passages. Electron microscopic observation revealed the presence of clusters of HAV particles of 27 nm mainly in the lysosomal vesicles as described in the previous reports (Shimizu *et al.*, 1978, 1982; Shavrina-Asher *et al.*, 1987). Moreover, crystalline arrays of HAV particle out of vesicles were observed by us in the cytoplasm. The specific identification of HAV particles was confirmed by indirect immunocolloidal gold staining. Therefore, it was concluded that HAV similarly to other enteroviruses propagated in the cytoplasm and was taken up into the lysosomal vesicle. In the present study, a HAV strain passaged in marmosets and a wild strain of HAV were inoculated into a new cell line derived from the cortex of the kidney of cynomolgus monkeys, which was hormone-sensitive and possessed specific renal function. HAV was confirmed to propagate favorably in this cell line. Therefore, it is considered that this cell line may be extremely useful in studies on the molecular biology of HAV propagation as well as on the pathogenesis of hepatocellular injury.

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