Acta virol. **30** : 463—467, 1986

PROPAGATION OF HEPATITIS A VIRUS IN HUMAN DIPLOID FIBROBLAST CELLS

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Received March 25, 1986

Summary. — Human hepatitis A virus (HAV) was propagated in human diploid fibroblast cultures (2BS cells) in vitro. Replication of the virus was followed by immunofluorescent staining (IF), indirect ELISA, and by immune electron microscopy. When 2BS cells were inoculated with faecal extracts containing HAV, synthesis of hepatitis A antigen (HAAg) could be detected in the cytoplasm by IF. Its concentration reached a maximum at four weeks post-inoculation. Measured by solid-phase indirect ELISA, the positive/negative (P/N) ratio for HAAg reached values of up to 7.7. The identity of newly synthesized virus particles with HAV was established by immune electron microscopy, IF-blocking, and neutralization with human convalescent serum. Infected cells showed no signs of a specific cytopathic effect. Two of the virus strains propagated in 2BS cells may prove useful as a source of antigen for serologic tests; one of them might be a candidate strain for HAV vaccine.

Key words: *Hepatitis A virus*; *HAV replication*; *faecal extracts*; *2BS human diploid cells*; *immunofluorescence*; *ELISA*

Introduction

In general, efficient propagation of a virus in vitro in cell cultures is a prerequisite for detailed experimental analysis as well as for production of viral antigen for diagnostic and vaccine purposes. Hepatitis A virus replication in cell cultures could be achieved only very recently. In 1967, HAV had been transmitted to marmosets (Deinhardt *et al.*, 1967); in 1973, small spherical particles with the antigenic properties of HAV were first discovered in the faeces of patients suffering from hepatitis A, and only in 1979 Provost and Hilleman reported successful propagation of the marmoset-adapted HAV strain CR 326 in foetal rhesus monkey kidney cells. More recently, HAV has been adapted to human diploid cells such as human embryo fibroblasts (Gauss-Müller *et al.*, 1981), to diploid human embryonic lung (MRC-5) cells (Siegl *et al.*, 1984) as well as to SL7 (2BS) cells (Hu *et al.*, 1984).

We succeeded in propagating HAV directly from faecal extracts in human diploid fibroblasts (2BS cells). Here we describe the growth characteristics of our isolates and discuss their usefulness as vaccine strain candidates.

Materials and Methods

Faecal specimen. Faecal specimens were collected during an epidemic of hepatitis A in the suburban areas of Shenyang in 1978—1982. In some of the faeces, HAV particles could be detected by immune electron microscopy and the presence of HAAg was demonstrated by means of immune adherence haemagglutination (Wang *et al.*, 1979). HAV particles of one of the samples banded at a density of 1.34 g/ml in CsCl density gradient centrifugation and could be detected in the respective gradient fractions by RIA (Abbott, North Chicago). Two control specimens were collected from children who failed to contact hepatitis A in primary school.

Of the faeces shown to contain HAAg, 10% extracts were prepared in phosphate buffered saline (PBS), pH 7.4. The suspension was centrifuged at 3 000 rev/min for 30 min and then at 8 000 rev/min for the same period. The final supernatant was passed through 0.3 μm filters and stored at −30 °C for further use.

Cell cultures. Serial cultures of human embryonic lung (2BS) cells (Beijing Institute of Bioproducts) were used to isolate HAV. These cells were grown either on coverslips in culture tubes with 2 ml of medium or in 50 cm^2 flasks with 10 ml medium, which consisted of Eagle's MEM supplemented with 0.5% (v/v) lactalbumin hydrolysate (Oxoid), 10% heat inactivated newborn bovine serum, 2 mmol/l L-glutamine, 50 μg/ml of kanamycin, 100 μg/ml of streptomycine, and 100 units/ml of penicillin G. The pH was kept at 7.4—8.0. Cultures were maintained either in closed flasks or were incubated under 5% CO_2. Maintenance medium contained only 2% newborn bovine serum (NBS).

Virus propagation. Primary isolation. Cultures of 2BS cells in tubes or flasks were washed twice with Hank's balanced salt solution. The 10% stool suspension was diluted 1 : 10 or 1 : 50 in the culture medium and adsorption was allowed to proceed at 37 °C for 4 hr. Thereafter, the cultures were incubated at 32 °C. The medium was changed at weekly intervals.

Serial passage. As soon as infected cultures reached of confluency, they were harvested by trypsinization and split at 1 : 2 ratio. Coverslips were assayed weekly for the presence of HAAg by IF and/or ELISA.

Harvesting of infected cultures. As soon as HAAg could be detected by IF, cells were harvested by treatment with 0.25% trypsin, 0.03% EDTA for 1 min and collected in maintenance medium without NBS. After two cycles of freezing and thawing, and after ultrasonication for 30 sec, the extracts were centrifuged at low speed for 30 min and stored frozen for further use.

Assay of HAAg. Indirect immunofluorescent assay. Infected coverslip cultures were rinsed with PBS and fixed in cold acetone for 1 min. Convalescent serum from a patient suffering from hepatitis A was used as the first antibody. Sheep anti-human IgG conjugated with fluorescein isothiocyanate (Shanghai Institute of Bioproducts) was used as the second antibody. The coverslips were examined in VANOX microscope.

Immunofluorescence blocking assay. Fixed coverslip cultures were reacted with either mouse anti-HAV serum or normal mouse serum at dilutions of 1 : 4 before human hepatitis A convalescent serum and the second, conjugated antibody were added. Blocking of positive IF by mouse anti-HAV serum was regarded to prove the presence of HAAg and the specificity of IF tests.

Indirect ELISA. The method of Dong *et al.* (1984) was used. Wells of polystyrene microplates (40 well plates, Shanghai) were coated at 4 °C overnight with twofold serial dilutions of HAAg harvested from infected 2BS cultures. After washing three times with 0.02 mol/l PBS-Tween, either human anti-HAV IgG or negative human control serum was added to the wells and the plates were further incubated for 2 hr at room temperature. The plates were washed again and bound antibody was tested by addition of peroxidase conjugated sheep anti-human IgM or IgG (Shanghai Institute of Bioproducts). After incubation for further 2 hr at room temperature and repeated washing, the peroxidase substrate OPD was added for 15—20 min at room temperature. The reaction was stopped by addition 1 N sulphuric acid and the optical density was measured at 492 nm in a spectrophotometer. A positive/negative (P/N) ratio of 2.1 or greater was considered of being indicative for the presence of HAAg.

Immune electron microscopy. Immune electron microscopy was performed as described by Feinstone *et al.* (1973) with a slight modification. 0.45 ml of cell culture derived HAAg were mixed with 0.05 ml of a 1 : 80 to 1 : 100 dilution of human hepatitis A convalescent serum (or anti-HAV IgG), incubated at 37 °C for 1 hr, and finally, at 4 °C overnight. The mixture was centrifuged at 4 °C and 14 000 rev/min for 90 min. The aggregates in the pellet were transferred to an electron microscope grid, negatively stained with phosphotungstic acid, pH 6.8—7.0, and examined in a Hitachi electron microscope.

Serum neutralization test. 100 $TCID_{50}$ of cell culture adapted HAV (second passage) were incubated with ten fold serial dilutions of either human pre-infection or convalescent serum. After incubation at 37 °C for 1 hr, 0.1 ml of the reaction mixture in 2 ml of growth medium were inoculated onto 2BS coverslip cultures. Replication of HAV was determined by IF; the neutralization titres were read within 4—6 weeks.

Heat treatment. The HAV harvested from cell cultures was heated at 60 °C for 10 min; 0.1 ml aliquots of each sample were inoculated into 2BS coverslip cultures and cells were assayed for HAAg by IF after 4 weeks of incubation.

Results

Three to four week after inoculation, cultures infected with extracts of two faecal samples proved positive for HAV replication when tested by IF. A large number of cells in such cultures contained apple-green fluorescent granules in the cytoplasm and, particularly, in the perinuclear region (Fig. 1). In cultures incubated for 39 days and consisting to about 75% of infected cells, fluorescence could be specifically blocked by addition of mouse anti-HAV serum. Normal mouse serum, on the other hand, failed to interfere with the IF staining reaction. Infected cells appeared normal under the light microscope, i. e. replication of HAV apparently failed to induce cytopathology. In parallel to the positive IF staining reactions in coverslip cultures, harvests of 2BS cell cultures infected with the same two faecal extracts also proved positive for HAAg by ELISA. As examplified in Table 1, the positive/negative ratio was in the range of 7—8.

To establish the identity of cell culture derived antigen with HAAg, harvests were examined by immune electron microscopy. In accordance with the known morphology of HAV (Provost and Hilleman, 1979), this test revealed aggregates of small spherical particles with a diameter of 27—30 nm (Fig. 2). Moreover, harvests contained infective virus which could be readily passaged growing to titres of about 10^6 $TCID_{50}$/ml already after 2 to 3 passages in vitro. Infectivity of virus harvested from the second passage

Table 1. Assay by ELISA of antigen synthesized in 2BS cell cultures infected with two HAV strains

Strain	OD value	P/N
SY-Y 8	0.40	8.3
SY-M 11	0.35	7.2
control	0.048	1.0

Table 2. Infectious HAV in harvest of 2BS cell cultures infected with two faecal samples of derived virus strains

Strain	Passage No. (days)	$TCID_{50}$/ml (log_{10})
SY-Y 8	3 (29)	6.6
SY-M 11	2 (23)	6.0

could be neutralized by human convalescent anti-HAV serum but not by incubation with human pre-infection serum (Table 2).

Finally, the ability of cell culture adapted virus to replicate in 2BS cells could not be abolished by heating the inocula at 60 °C for 10 min.

Discussion

In accordance with the observations of several authors (Frösner *et al.*, 1979; Flehmig, 1980; Gauss-Müller *et al.*, 1981; Hu *et al.*, 1984; Siegl *et al.*, 1984), we were able to isolate HAV directly from human faecal specimens in cell cultures. The identity of the agent recovered in cell cultures with HAV was established on the basis of known physicochemical properties of the latter virus such as size and resistance to incubation at 60 °C. Moreover, virus particles derived from cell culture could be precipitated and infectivity could be neutralized with human hepatitis A convalescent serum whereas neither precipitation nor neutralization was observed with pre-infection serum. The reaction of intracytoplasmic viral antigen with human convalescent serum in IF staining reactions could be also blocked by mouse anti-HAV serum.

In some of the attempts to isolate HAV directly from faecal samples and to propagate the virus in vitro, the so-called Alexander (PLC/PRF/5) cell line (Alexander *et al.*, 1978) has been used successfully (Frösner *et al.*, 1979; Hu *et al.*, 1982). This cell line was derived from a human hepatoma, contains multiple copies of the hepatitis B virus genome, and continues to synthesize hepatitis B surface antigen. These characteristics restrict the thus propagated HAV particles to the use as antigen in serologic tests in spite of the fact that HAAg can be obtained in considerable quantities from PLC/PRF/5 cell cultures. HAV and HAAg synthesized in human diploid fibroblast cells such as 2BS cells, however, offer themselves both as potent antigen for diagnostic tests and as substrate for the development of a suitable vaccine against human hepatitis A.

Acknowledgement. We are grateful to Prof. Dr. G. Siegl for his help in the preparation of this manuscript.

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Explanation to Figures (Plate LV):

Fig. 1. Immunofluorescent staining of 2BS cell cultures infected with HAV strain SY-Y8 and incubated for 30 days at 32 °C. The inoculum had previously been passaged 3 times in the same culture system.

Fig. 2. Immune electron microscopy of HAV particles harvested from 2BS cell cultures 60 days after infection with HAV strain SY-Y 8 (2nd passage at 32 °C).