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ISOLATION AND PROPAGATION OF HEPATITIS A VIRUS IN HEPATOMA CELL CULTURES

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Summary. — Hepatitis A virus (HAV) was isolated directly from human faeces in PLC/PRF/5 cells. In the first passage cell-bound and supernatant viruses were found by immune electron microscopy and by enzymeimmunoassay. Serial passaging of HAV in PLC /PRF/5 cells resulted in its adaptation to the cell line and in reduction of the incubation time. HAV was still detectable after 10 cell passages. Cell-bound as well as supernatant HAV were employed as antigens in anti HAV IgM-enzymeimmunoassay.

Key words: hepatitis A virus; virus propagation; PLC/PRF/5 cells

Introduction

During the last five years hepatitis A virus (HAV) has been successfully propagated in several different types of cell cultures. The first propagation in vitro was reported by Hilleman *et al.*, (1979) using a HAV strain passaged 31 times in marmosets. Later several authors were able to isolate and propagate HAV even directly from the faeces of human hepatitis A cases (Froesner *et al.*, 1979; Flehming, 1980; Daemer *et al.*, 1981; Provost *et al.*, 1981). In all these studies no cytopathic effect was observed and cell-bound hepatitis A antigen was detected by immunological methods, such as immunofluorescence, solid phase radioimmunoassay or immune electron microscopy. The development of detectable HAV in the infected cells takes approximately 4 weeks, but this interval can be reduced by serial virus passages.

A much more rapid appearance of intracellular HAV was observed by Balayan and Locarnini (Balayan *et al.*, 1979; Locarnini *et al.*, 1981). These authors detected intracellular HAV by immunofluorescence within 1 to 4 days after inoculation. There are discrepancies concerning the release of HAV from infected cells into the supernatant fluid. While most investigators failed to detect HAV in cell culture supernatants, Flehmig demonstrated HAV in the culture medium of infected Frhk-4 cells and Crane in the supernatant of hepatoma cells (Flehmig *et al.*, 1981; Crane *et al.*, 1983). The present report describes the isolation and propagation of HAV in PLC/PRF/5 cells directly from infected stools of three patients with acute hepatitis A infection as well as the detection of cell-bound and supernatant HAV in serial cell passages.

Materials and Methods

Hepatitis A virus. During an epidemic outbreak of hepatitis A stool samples were collected in the first days of acute illness from the following three patients: IH-26 (female, 26 years), BR-51 (male 51 years), and WR-61 (male 23 years). Faecal extracts were prepared as 20% suspensions in phosphate buffered saline (PBS) supplemented with penicillin (200 IU/ml) and streptomycin (200 μg//ml) and stored at −20 °C. These stool suspensions were tested for the presence of HAV particles by immune electron microscopy (IEM) and for HAV antigen by enzyme immunoassay (EIA) using human convalescent serum with an anti-HAV titre of 3 000 (as detected by radioimmunoassay HAVAB, Abbott).

Cell culture. Cell line derived from a human primary liver cell carcinoma (PLC/PRF/5) was used in this study. These cells were kindly provided by Prof. Desmyter, University of Leuven, Belgium. The doubling time of the PLC/PRF/5 cells was one week in Eagle's minimal essential medium (MEM) containing 5% foetal calf serum (FCS) and 5% neonatal calf serum (NCS), 100 IU/ml penicillin and 100 μg/ml streptomycin. The cells were grown in 18 cm flasks at 36.5 °C.

Hepatitis A virus isolation and propagation. For virus isolation the 20% stool suspencions were diluted 1 : 10 in MEM without serum. After removing the medium from cell cultures, the cell monolayer was infected with 0.5 ml of virus suspension. Following 4 hr incubation at 37 °C, 4.5 ml MEM with 2% FCS were added and the incubation was continued at 37 °C. The maintenance medium was changed once a week. Samples of supernatant fluids and of infected cells including noninfected controls were harvested for further investigations. The supernatant fluids were clarified by centrifugation, cells were extracted by three cycles of freezing and thawing and suspended in 0.5 ml MEM. Both materials were tested by EIA and IEM without further concentration. Passaging of HAV in the PLC/PRF/5 cell line was carried out by serial inoculations of supernatant HAV (HAV-s) as well as of cell-bound HAV (HAV-c) in a 1 : 10 dilution onto monolayers of PLC/PRF/5 cells as described above.

Immune electron microscopy was performed with the supernatant from infected cells and with the cell culture extract. 200 μl of the suspension to be tested for HAV were mixed with 100 μl of the human convalescent serum, incubated for 1 hr at 37 C and overnight at 4 °C. These mixtures were centrifuged for 2 hr at 15,000 rev/min and the pellets were resuspended in 100 μl PBS. Carbon-coated grids were overlaid with a drop of the suspensions, face downward, picked up, stained in the same manner with phosphotungstic acid (PTA) pH 7.0 and examined in Siemens EM 102 electron microscope.

Enzyme immunoassay (EIA). HAV antigen was tested in microtitre plates (Dynatech). Briefly, wells were coated with anti-HAV IgG by overnight incubation at 4 °C. Then the plates were washed three times and 50 μl of HAV suspension to be tested was added. Plates were then incubated for 20—24 hr at 4 °C, washed, and the conjugate (human anti-HAV IgG labelled with horse-radish peroxidase) was filled into the wells. Following 2 hr incubation at 37 °C the orthophenylene diamine/H_2O_2 substrate was added. The reaction was stopped after 30 min incubation at room temperature by adding 2 mol/l H_2SO . The specificity of the assay was confirmed by blocking with human anti-HAV positive sera.

Results

All three investigated stool samples contained the typical HAV particles in diameter of 27 nm (Fig. 1). The antigen titres by EIA were 16 (IH-26, 64 (BR-51) and 16 (WR-61). According to the accepted sensitivity of the EIA and IEM, these titres correspond to the virus concentrations higher than 10^5—10^6 particles/ml in the sample. HAV-c was detected by EIA and IEM 3 weeks post-inoculation (p.i.) (Fig. 2-I). The virus reached a peak concentration from 6th to 10th week p.i., the EIA titre being 64. Decreasing concentrations of HAV-c were still demonstrable in weeks 12 and 15 p.i. Infected hepatoma cells showed no specific cytopathic changes during the entire 15 weeks investigation period. Free HAV first appeared in the medium of infected cell cultures 4 weeks p.i. The kinetics of virus release from the infected cells

corresponded to those of the appearance of HAV-c. The titre of 4 of HAV-by EIA was much lower than that of HAV-c (Fig. 3). Typical HAV particles in the supernatant of infected cultures were also demonstrated by IEM (Fig. 2-II).

Several times passaging of HAV-c through hepatoma cells resulted in an adaptation of HAV to these cells and reduced the time of the first HAV-c detection to one week p.i. HAV-c and HAV-s were still detectable after 10 cell passages (Fig. 4). Both HAV-c as well as HAV-s could be employed as antigens in our diagnostic tests. In that respect there were no differences between stool-derived HAV and cell culture-derived HAV. The HAV-s production of one culture flask, containing approximately 2×10^6 cells, was sufficient for 1,200 anti-HAV IgM tests as used in our laboratory. Harvests of the supernatant virus (HAV-s) could be repeated for at least 4 weeks.

Discussion

As known for many viruses propagated in cell culture, detection of the virus and its components depends on the sensitivity of the methods employed. After years of failure, Froesner succeeded in propagating HAV directly from

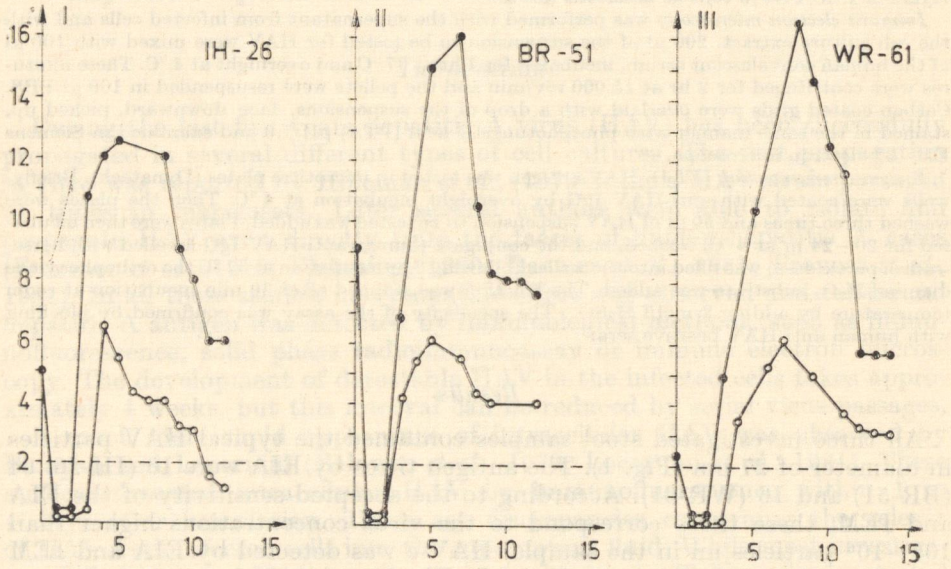

Fig. 3.

Detection of HAV-c (●———●) and HAV-s (○———○) antigens produced in hepatoma cells in the first passage from stool samples

Interrupted line (- - -) cut off.

I — IH-26; II — BR-51; III — WR-61

Abscissae: weeks p.i.; ordinates: P/N ratio in EIA.

stool samples in a hepatoma cell line (Froesner *et al.*, 1979). Since then HAV propagation was achieved in various cell cultures, but in most systems initial virus production is slow and proceeds without virus release into the medium. In 1981 Gaus-Müller reported an atypically long incubation period (120 to 210 days) of HAV in human embryo fibroblasts (Gauss-Müller *et al.*, 1981).

In certain hepatoma cells HAV is only detectable after a long lag period between 4 and 7 weeks (Deinhardt *et al.*, 1981; Crane *et al.*, 1983). Serial cell passages reduce the lag phase to one week. In the first passage in PLC/PRF/5 cells HAV-c and HAV-s were detected 3 and 4 weeks p.i., respectively. This relatively short lag period was independent of the virus concentration in the original stool sample. HAV-c and HAV-s were demonstrable and proved to be infectious in all passages. Our findings, however, do not allow any conclusions about the mechanism of virus release from the cells into the culture medium.

In order to obtain a large amount of HAV grown in cell culture, it is necessary to use a suitable cell type and an appropriate virus inoculum. The described system is perfectly adequate for diagnostic purposes. The simultaneous production of HBsAg by PLC/PRF/5 hepatoma cells does not affect the specificity of the HAV test. All noninfected controls were negative for HAV in EIA. Anders used the culture fluids of a microcarrier Frhk-4 cell culture system to produce HAV antigen for diagnostic tests Anders *et al.*, 1984). As one might expect, the amount of virus produced in microcarriers is three times higher than in a monolayer system without microcarrier.

Though HAV has recently been classified as an enterovirus, little is known about its interactions with susceptible cells. The investigations of Gauss-Müller *et al.* (1984) indicate, that HAV has no major effect on host cell metabolism. The possibility of cultivating HAV in different cell systems is an important prerequisite for studying the molecular biology of HAV replication.

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Explanation of Figures (Plates LXXIII—LXXV):

Fig. 1. Immune complex of 27—32 nm HAV particles, stool sample WR-61 (magn. × 240,000)

Fig. 2. Immune complex of HAV particles harvested from the first passage in hepatoma cells, stool sample WR-61
I) cell-bound HAV (× 200,000)
II) supernatant HAV (× 200,000)
Identical results were obtained with stool samples BR-51 and IH-26
BR-51 and IH-26

Fig. 4. Immune complex of HAV particles from the 10th passage in hepatoma cells, stool sample WR-61
I) HAV-c (× 240,000)
II) HAV-s (× 240,000)
Identical results were obtained with stool samples
BR-51 and IH-26