Isolation and identification of hepatitis E virus in Xinjiang, China

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This paper describes isolation and identification of a virus (termed strain 87A) which has the cytopathic effect and haemagglutination properties of hepatitis E virus (HEV). This virus was isolated by tissue culture from the faeces of a patient with acute non-A, non-B enteric hepatitis in Xinjiang, China. The isolated virus was neutralized by acute phase sera obtained from other patients with acute non-A, non-B enteric hepatitis. The virus particles also could be specifically aggregated with acute phase sera from patients with known HEV hepatitis in China, Burma, India and the U.S.S.R., and with acute and convalescent sera from an HEV-infected chimpanzee. Crystalline arrangements of virus particles in the cytoplasm were observed by electron microscopy in ultrathin sections of infected cells. The sedimentation coefficient of the strain 87A virus particles in sucrose gradients was 176S. Purified virus particles revealed a protein band of about 76K on SDS–PAGE and Western blotting. The evidence indicates that the strain 87A virus is an HEV. Our ability to propagate HEV in cell culture should facilitate research on this hepatotropic virus.

Introduction

Hepatitis E (HE; enterically transmitted non-A, non-B hepatitis) was first described in India in 1980 (Khuroo, 1980). Subsequently documented epidemics of HE have been described in the Soviet Union, Nepal, Burma, Pakistan, Borneo, Somalia, Sudan, Ivory Coast, Algeria and Mexico (Gust & Purcell, 1987). An epidemic also occurred in 1986 to 1988 in Xinjiang, China (Cao et al., 1989). These outbreaks primarily affect young to middle-aged adults and result in a high mortality rate (approaching 20%) in infected pregnant women. Several investigators have reported 27 to 34 nm virus particles in stools of acutely infected cases (Bradley, 1990). Balayan et al. (1983) were the first to demonstrate experimental transmission of HE virus (HEV) to human volunteers and cynomolgus macaques. Other investigators (Andjaparidze et al., 1986; Bradley et al., 1987; Panda et al., 1989) reported transmission of HEV to African green monkeys, chimpanzees and rhesus monkeys, and infected primates have been shown to shed 27 to 34 nm virus particles. However, HEV has not been propagated in tissue culture (Bradley, 1990). The physicochemical properties of this viral agent have therefore not been properly studied and specific diagnosis of HEV remains a problem. The taxonomic status of HEV also has not been determined. We report here a strain of HEV in China isolated and propagated in cell culture.

Methods

Specimen. During an outbreak of enteric non-A, non-B hepatitis in the Kashi region of Xinjiang, China in 1987, AMD, a 21-year-old female was suspected to be a case of HEV infection. Faecal specimens were collected on six occasions between 19 November and 9 December, 1987. She had complained of jaundice, anorexia and nausea since 27 November, 1987. The serum showed elevated alanine transaminase (ALT) levels (222 units), was positive for anti-hepatitis A virus (HAV) IgG, and negative for anti-HAV IgM, hepatitis B surface antigen and IgM against hepatitis B core antigen. Crude stool suspensions (collected on 19 November) were diluted to 20% (w/v) in PBS and were centrifuged at 2000 r.p.m. for 30 min; large numbers of virus-like particles were found in the supernatant by immunoelectron microscopy (IEM). Further treatment of the specimen was as previously described (Huang et al., 1990a).

Cell culture. Human embryo lung diploid cell strain 2BS, obtained from the Beijing Institute of Biological Products, China and the LLC-MK2 continuous cell line were used. Culture media and methods were as described elsewhere (Huang et al., 1990a).

Sera. Acute and convalescent sera from a patient with HE (Xinjiang; no. 109) were taken at an interval of 10 months and were kindly supplied by Dr H. Z. Xu. Acute sera from patients with hepatitis A and B were provided by Dr X. H. Yu. Sera from an experimentally HEV-infected chimpanzee (pre-inoculation, acute and convalescent) were supplied by Dr R. H. Purcell. Acute sera from patients infected with HEV from Burma, India and the U.S.S.R. were supplied by Dr A. G. Andjaparidze. Normal human sera were collected from the 307 Hospital, Beijing, China. All sera were inactivated at 56 °C for 30 min before testing.

Virus isolation. The treated specimen containing virus-like particles was inoculated onto 2BS and LLC-MK2 cells that had been pre-treated
with 10 μg/ml trypsin for 10 min. The cells were then incubated at 37 °C for 2 h for viral adsorption and the supernatant was decanted. The cell monolayers were washed with Hank's" solution. Eagle's medium containing 2% inactivated calf serum, 30 mM-MgCl₂, 100 units/ml penicillin, 100 μg/ml streptomycin and 50 units/ml kanamycin was added as maintenance medium. The inoculated tissue cultures were incubated in roller drums at 35 °C and were observed for c.p.e. daily for 1 week. Two additional blind passages were made. Virus-containing cultures were serially passaged as described above.

Haemagglutination (HA) test. PBS was used as the diluting solution and human O erythrocytes were used for HA. The routine method required incubation at 37 °C for 1 h.

Neutralization test (NT). NT was performed using 1:10 dilutions of sera obtained from patients with acute HE and mixed with an equal volume of different dilutions of the strain 87A virus. After incubation at 37 °C for 1 h, the NT mixture was inoculated onto cell monolayers in two tubes or four microplate wells. Cultures were observed daily over a period of 7 days and a neutralization index was calculated [expressed as the ratio of control virus titre (TCID₂₀) over the virus titre of the neutralization mixture].

Electron microscopy (EM). The cultured cells infected with the strain 87A virus (second or eighth passage) were harvested, fixed, dehydrated, embedded, sectioned, stained and then observed under the electron microscope.

IEM. Suspensions of fifth to tenth serially passaged strain 87A-inoculated cells (c.p.e.) were frozen and thawed three times. After centrifugation (1500 r.p.m., 30 min), the supernatant was mixed with acut serum obtained from HE patients and control subjects (diluted 1:10 to 1:80) in equal volumes, incubated for 1 h at 37 °C and then incubated overnight at 4 °C. The samples were then prepared and stained with 2% phosphotungstic acid pH 7.2 and observed under a Philips 400T electron microscope.

Virus purification. Strain 87A virus was purified from infected cell cultures by precipitation with 6% polyethylene glycol 6000 and by ultracentrifugation through 10 to 40% sucrose gradient in PBS using a Beckman 28T1 rotor (25000 r.p.m., 3 h, 10 °C). Serial 0.5 ml fractions were collected and the sucrose concentration and HA activity of each fraction were recorded.

SDS-PAGE and Western blotting. A purified suspension of the isolated strain 87A virus and one of enterovirus echovirus type 13 (EV-13) as well as normal cells were analysed by the SDS-PAGE method (Han et al., 1989). After separation by SDS-PAGE the proteins were transferred to nitrocellulose papers at 20 mA for 6 h. The proteins were submerged in 3% chroic protein for 4 °C overnight. They were then incubated with 1:40 acute patient serum as well as with normal human serum for 2 h at 37 °C. These nitrocellulose preparations were washed with 0.015 M-PBS four times, and were treated with 1:400 horseradish peroxidase-conjugated anti-human μ chain monoclonal antibody (supplied by Dr Z. D. Li) for 1 h at 37 °C. After washing they were reacted with a diaminobenzidine-H₂O₂ solution for 10 min at room temperature. The Mr values of proteins were calculated by comparison with Mr standards.

Results

Isolation of virus

Monolayers of 2BS and LLC-MK2 cells in culture were inoculated with stool samples obtained from a patient with acute non-A, non-B enteric hepatitis in Xinjiang. By the third day of the second passage in the 2BS cell line, c.p.e. was observed. The characteristics of c.p.e. produced by virus strain 87A included rounding and then breaking of the cells, and finally destruction of the cell monolayer (Fig. 1). Consistent c.p.e. could be seen during serial passages. Haemagglutination could be observed from the fifth passage onwards. Cytopathic effects could not be detected by the third passage in the LLC-MK2 cell line or in normal cells. The positive results in 2BS cells were reproducible.

Serum neutralization test

Virus of strain 87A exhibited a high neutralization index only when acute phase sera from patients with acute non-A, non-B enteric hepatitis (including patient no. 109) from Xinjiang were tested. The neutralization index was very low or nil when tested with convalescent serum from patient no. 109 or with sera from patients with hepatitis A and normal controls (Table 1). The results suggest that the strain 87A virus was the causative agent responsible for the non-A, non-B hepatitis epidemic in Xinjiang in 1986 to 1988.

### Table 1. Neutralization of strain 87A virus

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Neutralization index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute serum (no. 109; Xinjiang)</td>
<td>589</td>
</tr>
<tr>
<td>Convalescent serum (no. 109; Xinjiang)</td>
<td>6</td>
</tr>
<tr>
<td>Acute serum (no. 2; Xinjiang)</td>
<td>1000</td>
</tr>
<tr>
<td>Acute serum (no. 3; Xinjiang)</td>
<td>1000</td>
</tr>
<tr>
<td>Acute serum (no. 8; Xinjiang)</td>
<td>1000</td>
</tr>
<tr>
<td>Acute serum from a patient with HAV infection</td>
<td>66</td>
</tr>
<tr>
<td>Pooled serum from healthy adults</td>
<td>10</td>
</tr>
</tbody>
</table>

### Table 2. Identification of strain 87A virus by IEM

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Number of sera tested</th>
<th>IEM titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chimpanzee sera</td>
<td>1</td>
<td>&lt;1:3</td>
</tr>
<tr>
<td>Pre-inoculation</td>
<td>1</td>
<td>1:3</td>
</tr>
<tr>
<td>Acute</td>
<td>1</td>
<td>1:3</td>
</tr>
<tr>
<td>Convalescent</td>
<td>1</td>
<td>1:3</td>
</tr>
<tr>
<td>Hepatitis E acute sera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>China</td>
<td>10</td>
<td>1:40-1:80</td>
</tr>
<tr>
<td>Burma</td>
<td>1</td>
<td>1:80</td>
</tr>
<tr>
<td>India</td>
<td>1</td>
<td>1:20</td>
</tr>
<tr>
<td>U.S.S.R.</td>
<td>1</td>
<td>1:80</td>
</tr>
<tr>
<td>Hepatitis A acute sera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>China</td>
<td>5</td>
<td>≤1:10</td>
</tr>
<tr>
<td>Hepatitis B acute sera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>China</td>
<td>5</td>
<td>≤1:10</td>
</tr>
<tr>
<td>Normal human sera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>China</td>
<td>5</td>
<td>≤1:10</td>
</tr>
</tbody>
</table>
Identification by IEM

Strain 87A virus could be specifically aggregated with low dilutions of acute and convalescent sera obtained from a chimpanzee experimentally infected with HEV and with acute phase sera from HE patients from China, Burma, India and the U.S.S.R. (Table 2). Bridging and coating of antibody were very clearly seen by IEM. In contrast, aggregation was not seen with pre-inoculation serum from the same chimpanzee nor with sera from patients with hepatitis A, hepatitis B and normal controls. These results also suggest that the strain 87A virus is an HEV and is similar to those found in several other countries. Two forms of the virus particles were found by EM: empty particles of 27 nm in diameter and...
full particles of 30 to 34 nm in diameter. The sizes of the purified virus particles were uniform but the particle surfaces appeared rough and irregular; the electron density was not homogeneous (Fig. 2).

**EM observations**

Characteristic cytopathic changes, such as vacuoles and membrane-bound vesicles, were found in the cytoplasm of 2BS cells infected with the strain 87A virus (Fig. 3a). Marked morphological changes in the nucleus were also present. On higher magnification, the strain 87A virus particles formed crystalline arrays in the cytoplasm (Fig. 3b). The size and morphology of the virus particles were similar to those observed in stool specimens from the reference patient.

**Determination of sedimentation coefficient of the strain 87A virus**

Table 3 shows the different sedimentation coefficients in sucrose gradients between the strain 87A virus and EV-13. The strain 87A virus particles sedimented faster (in a sucrose concentration of 29.0%) than EV-13 (25.8%) in parallel runs. The calculated sedimentation coefficient of the strain 87A virus was approximately 176S (in contrast to 157S for EV-13). The virus particles in the peak fraction of each virus gradient were studied by negative staining. The results showed that the diameter of EV-13 particles is about 27 nm but the diameter of strain 87A virus particles is 30 to 32 nm.

**Virus structural protein analysis**

Strain 87A virus particles were separated by SDS-PAGE and then immunoblotted with 1:40 acute-phase serum from a patient with HEV infection (Fig. 4, lane 2). Four protein bands were found, at 76K, 44K, 41K and 37.5K. Immunoblots of EV-13 with 1:40 acute serum
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Fig. 4. Structural protein analysis of the strain 87A virus by immunoblotting. Lane 1, Mr standards; lane 2, 87A virus + 1:40 acute serum from a patient with HEV infection; lane 3, 87A virus + 1:40 normal human serum; lane 4, EV-13 + 1:40 acute serum from a patient with HEV infection; lane 5, EV-13 + 1:40 normal human serum; lane 6, normal cell antigens + 1:40 acute serum from a patient with HEV infection; lane 7, normal cell antigens + 1:40 normal human serum.

Discussion

HE was first described in 1980 (Khuroo, 1980). Since then, investigators have carried out studies on animal transmission (Balayan et al., 1983; Andjaparidze et al., 1986; Bradley et al., 1987; Panda et al., 1989) and gene cloning (Reyes et al., 1990); however, to our knowledge no one has yet propagated HEV in tissue culture (Bradley, 1990). This paper describes the results obtained with a strain of HEV isolated from stool samples of a Chinese patient during the incubation period. The virus was successfully propagated in human embryo lung diploid cell culture. The size and morphological characteristics of this virus were similar to HEV described earlier by other investigators (Kane et al., 1984; Bradley et al., 1987; Panda et al., 1989). The successful isolation of HEV in our laboratory depended on three factors: the use of a very sensitive cell strain, the use of a stool specimen obtained during the incubation period and the addition of 30 mM-Mg$_2^+$ to the maintenance medium to protect labile virus particles.

In our laboratory two of four infected rhesus monkeys developed elevated ALT levels after inoculation with isolated strain 87A virus (sixth passage). Liver biopsy of the infected monkeys showed histopathological changes. Virus was reisolated from mixed faeces of the two infected animals and antibody titres increased by four- to 32-fold, in comparison to those before infection (Huang et al., 1990b). Inoculated suckling mice showed apparent jaundice (Huang et al., 1990b).

The isolated virus could be neutralized by patients’ sera. The purified virus particles could be specifically aggregated by antibody obtained from patients with known HEV in China, Burma, India and the U.S.S.R., as well as from experimentally HEV-infected chimpanzees. A. G. Andjaparidze also demonstrated that our isolated virus can cross-react with HEV in stools obtained from patients in the U.S.S.R. (H. M. Li, personal communication). These results strongly suggest that the strain of HEV isolated by us is related to those from abroad and that strain 87A virus is the aetiological agent of HE in Xinjiang.

Five pairs of acute and convalescent sera gave similar titres as determined by IEM (data not shown). These results were similar to those previously reported (Aran-kalle et al., 1988). The neutralization titre of a convalescent serum (no. 109) was lower than that of acute serum. This may be due to the fact that this serum was obtained 10 months after infection.

Structural protein analysis of HEV has not previously been reported. Purified 87A virus was analysed by SDS–PAGE and immunoblotting, and a major 76K protein was found. More detailed studies of this protein are required. The sedimentation coefficient of the strain 87A virus is similar to that reported by Bradley (1990), is different from that of echovirus (used as a reference), and is compatible with that of caliciviruses (Reyes et al., 1990).

This virus was very labile when stored between $-25$ and $-70^\circ$C (R. T. Huang, unpublished results). Morphologically the virus is spherical and unenveloped and in purified preparations it measures 30 to 32 nm in diameter with an irregular and non-homogeneous surface electron density. It contains a protein of about 76K on SDS–PAGE and Western blotting. All these biophysical characteristics suggest that HEV is similar to caliciviruses, as other investigators have suggested (Aran-kalle et al., 1988; Bradley et al., 1988). Reyes et al. (1990) have recently reported that HEV might be a new
virus of the Caliciviridae family, and Tam et al. (1991) suggested that HEV represents either the prototype member of an as yet unclassified novel virus family or perhaps a separate genus within the Caliciviridae. Success in cultivating HEV in a human cell line in our laboratory will facilitate further research on HEV.


References


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