Cell Culture of Sporadic Hepatitis E Virus in China

RUTONG HUANG,1,* DERONG LI,1 SHAOJING WEL2 QINGHONG LI,1 XITONG YUAN,1 LIQING GENG,4 XIAOYU LI,1 AND MINXIA LIU1

Beijing Institute of Microbiology and Epidemiology, Beijing 100850,1 and Institute of Infections Diseases, Guangzhou Municipal Infectious Diseases Hospital, Guangzhou 510060,2 China

Received 18 November 1998/Retumed for modification 28 January 1999/Accepted 25 May 1999

The isolation and identification of the 87A strain of epidemic hepatitis e virus (HEV) by means of cell culture have been described previously. This paper reports the successful isolation of a sporadic HEV strain (G93-2) in human lung carcinoma cell (A549) cultures. The etiology, molecular and biological properties, and serological relationship of this new strain to other, epidemic HEV strains are described. The propagation of both sporadic and epidemic HEV strains in a cell culture system will facilitate vaccine research.
at 70°C, reverse transcription and PCR were performed. Two sets of sense and antisense synthetic oligonucleotide primers for the HEV ET1.1 region were used for detection of the HEV genome. The sequences of the primers used in this study were as follows: F1, 5'-GCT ATT GAG GAG TGT GG-3' (positions 4459 to 4478); R1, 5'-CAG GCC CCC AAT TCT TCT-3' (positions 4876 to 4859); F2, 5'-GGG TGG ATC TTG CAG GCC-3' (positions 4522 to 4539); and R2, 5'-TTC AAC TTC AAG CCA CAG CC-3' (positions 4760 to 4741). The other reactions were the same as those previously described (10). The PCR products were analyzed by 1.5% agarose gel electrophoresis. In order to confirm the specificity of the segments amplified by PCR, Southern blotting was carried out with a 239-bp DNA fragment obtained from HEV strain 87A as a probe. The recovered DNA was ligated with the pGEM-T Easy Vector (Promega) and transformed in JM109. Positive clones were screened by PCR and identified with an ABI model 373A DNA sequencer. The homology of this part of the nucleotide sequence was compared among strains G93-1, G93-2, G93-3, and G93-4 and Xinjiang strain 87A (7).

RESULTS

Virus passage. Cell isolation stocks (0.1 ml; passage 1) of strains G93-1, G93-2, G93-3, and G93-4 were inoculated into A549 cells and incubated at 37°C. CPE was visible at day 2 postinoculation for all four strains. The cell rounding and monolayer destruction were typical characteristics of the CPE produced by the viruses (Fig. 1).

Conditions for virus culturing. Analysis with SAS software of virus propagation in A549 cells revealed that the best propagation conditions were 30 mM Mg2+, pH 7.2, and related to the virus inoculation dose (Table 1). Mg2+ at 30 mM was necessary and very important.

Cell sensitivity. Results of cell sensitivity testing revealed that 2BS and A549 cells were sensitive to strains G93-1, G93-2, G93-3, and G93-4 but that LLC-MK2 cells were not (Table 2).

Physicochemical properties. Nucleic acid type, ether sensitivity, acid (pH 3.0) resistance, and heat (56°C, 30 min) stability for passage 8 strains G93-1, G93-2, G93-3, and G93-4 were determined by a microculture titration method with A549 cells (Table 3). The results for strains G93-1, G93-2, G93-3, and G93-4 were similar; the viruses were unenveloped RNA virus particles not resistant to acid (pH 3.0) or heat (56°C, 30 min).

Electron microscopy observations. Ultrastructural changes were mainly found in the cytoplasm of an infected cell. Virus particles of the four strains examined were arranged in the form of a crystal. Clusters contained several to hundreds of particles. The virion was round, approximately 25 to 36 nm in diameter. The surface of the virus was irregular. Empty particles were embedded in the crystal structure of completely mature viruses. Viral inclusion bodies and vacuoles were observed near the crystal (Fig. 2).

Immunoelectron microscopy. The virus particles of strain G93-2 could be identified by use of serum from a rabbit immunized with strain G93-2 or 87A and mouse hybridoma ascitic fluid derived from strain 87A (Fig. 3). The virus particles were all aggregated into clusters. Antibody bridge and antibody coat were found occasionally in some particles. Although aggregates of virus particles could occur in both HEV strains with polyclonal and monoclonal antibodies, the numbers in clusters of strain G93-2 were smaller than those in clusters of strain 87A. The efficiency of capture of virus particles by monoclonal antibodies was also lower than that by polyclonal antibodies. A 1:40 dilution of serum could be used for the capture of particles, while only a 1:16 dilution of ascitic fluid could be used. These results showed that strain G93-2 in Guangzhou and epidemic strain 87A in Xinjiang are closely related serologically.

Partial genome determination. When RNA from strains G93-1, G93-2, G93-3, and G93-4 as the template was amplified with primers for the HEV ET1.1 region, a band of approximately 239 bp was observed by gel electrophoresis. The PCR results for these four strains were confirmed by Southern blotting with a 239-bp probe from HEV strain 87A. After cloning, nucleotide sequencing analysis of the PCR fragments derived

TABLE 1. Best conditions for propagating HEV strain G93-2 in A549 cells

| Test group | Mg2+ concn (mmol/liter) | pH | Inoculum dose (MOI) | CPE | TCID50/0.025 ml |
|------------|------------------------|----|-------------------|-----|----------------|
| 1          | 0                      | 6.8| 0.25              | ++  | 4.2            |
| 2          | 0                      | 7.2| 0.025             | +   | 3.5            |
| 3          | 0                      | 7.6| 0.0025            | --  | 1.5            |
| 4          | 30                     | 6.8| 0.025             | ++++| 5.67           |
| 5          | 30                     | 7.2| 0.0025            | ++++| 5.83           |
| 6          | 30                     | 7.6| 0.25              | ++  | 5.23           |
| 7          | 60                     | 6.8| 0.0025            | --  | 2.0            |
| 8          | 60                     | 7.2| 0.25              | ++  | 5.0            |
| 9          | 60                     | 7.6| 0.025             | --  | 2.5            |

a = no cells showing CPE; +, 25% of the cells show CPE; ++, 50% of the cells show CPE; ++++, 75% of the cells show CPE; +++++, 100% of the cells show CPE.
from the virus strains was done (Fig. 4). The nucleotide sequence homologies in this part of the genome were 79.9% between strains G93-2 and 87A and 100% among strains G93-1, G93-3, and G93-4 and strain 87A.

DISCUSSION

Four HEV strains were isolated from four patients with sporadic HE in Guangzhou, China. The results suggested that A549 cells could be used to isolate and cultivate HEV. In addition, some continuous cell lines, such as 2BS, Hep-G2, and PLC-PRF-5, derived from human lung or liver, are susceptible to HEV (4, 7, 11, 13). The CPEs in both the 2BS and the A549 cell lines were certainly produced by the HEV infection. Those CPEs could be specifically neutralized by antibodies derived from sera of patients with HE, antibody from immunized-rabbit serum, and mouse ascitic fluid (data not shown). HEV also could be cultivated in in vivo-infected primary macaque hepatocytes, but no CPE was observed (16). CPE occurrence may be mainly related to the in vitro cell culture system used for HEV.

There are two reasons for the successful culturing of HEV in both 2BS and A549 cells. First, the stool suspension should be precipitated with PEG. Second, 30 mM MgCl₂ must be added to the culture medium in order to increase the titers of virus and protect viral infectivity from inactivating factors. This is a very important approach to resolving the problem of few HEV particles in acute-phase specimens. Therefore, these virus strains are closely related serologically.

On the basis of full-length or partial nucleotide sequences of HEV reported in many developing countries or areas in Asia since 1990, many scientists consider that the homology of HEV strains in Asia is comparatively high and that there is 75% homology between HEV strains in Asia and Mexico. Therefore, HEV may have two different subtypes or genotypes. In this report, a portion of the sequence of HEV strains G93-1, G93-3, and G93-4 was similar to those of Xinjiang strain 87A in China and a Burmese strain, but strain G93-2 was different from Xinjiang strain 87A, the Burmese strain, and a Mexican strain, with homologies of 79.9, 79.9, and 77.4%, respectively. A recent study suggested that a portion of the sequence of strain G93-2 has 99.2% homology with fragments from serum from a patient with HE (China X-S1) in Xiamen, which is near

| Virus strain (passage) | Prepassage | Postpassage in the following cells: |  |
|-----------------------|------------|----------------------------------|---|
|                        | A549  | 2BS  | LLC-MK₂ | |
| G93-1 (4)             | 5.5   | 5.83 | 5.5    | 0  |
| G93-2 (4)             | 4.67  | 5.67 | 5.33   | 0  |
| G93-3 (4)             | 5.5   | 5.67 | 6.0    | 0  |
| G93-4 (4)             | 5.5   | 5.22 | 5.77   | 0  |

* The titer was determined before and after three generations in A549 cells.

| Test group | Titer (TCID₅₀/0.025 ml) of the following virus strain: |
|------------|--------------------------------------------------------|
|            | G93-1 | G93-2 | G93-3 | G93-4 |
| Nucleic acid | 6.3   | 6.3   | 6.8   | 6.5   |
| Ether       | 6.3   | 6.4   | 6.4   | 6.4   |
| Acid (pH 3.0) | 1.3   | 1.0   | 1.3   | <1.0  |
| Heat (56°C; 30 min) | <1.0  | <1.0  | <1.0  | <1.0  |
| Virus control | 6.3   | 6.5   | 6.8   | 6.7   |

* The titer was determined in A549 cells.
Guangzhou (8). Thus, there is a new HEV genotype in China besides the Burmese and Mexican genotypes. We expressed this view in 1995 (10). Later, other investigators reported similar results (5, 17, 18). Recently, American scientists reported that strain US-1 was not similar to Asian strains or to the Mexican strain (14) and that Moroccan strains were close to Asian strains (3). Consequently, the sequences of HEV from various areas showed that the differences among HEV strains correlate with geographic location.

HEV has been provisionally described as a Calicivirus-like virus (12, 15). The size, morphology, and physicochemical properties reported in this study strongly support the notion that it is a member of the Caliciviridae.

Our results may provide candidate strains for the study of a Chinese HE vaccine. On the assumption that there might be only one serotype of HEV in Asia, selecting strain 87A or the four Guangzhou strains as candidate strains for a vaccine will be of significance for the prophylaxis and treatment of HE.

ACKNOWLEDGMENTS

We thank N. Q. Chen, J. J. Wang, and X. B. Xia, Beijing Institute of Microbiology and Epidemiology, for assistance in this study and X. K. Ma for proofreading.

REFERENCES

1. Aye, T. T., T. Uchida, X. Ma, F. Iida, T. Shikata, M. Ichikama, T. Rikihisa, and K. M. Win. 1993. Sequence and gene structure of the hepatitis E virus isolated from Myanmar. Virus Genes 7:95–110.
2. Bradley, D. W. 1992. Hepatitis E: epidemiology, aetiology and molecular biology. Rev. Med. Virol. 2:19–28.
3. Chattenjee, R., S. Tsarev, J. Pillot, P. Coursaget, S. U. Emeron, and R. H. Purell. 1997. African strains of hepatitis E virus that are distinct from Asian strains. J. Med. Virol. 5:139–144.
4. Chen, W. R., X. Tian, Z. Y. Bao, J. Y. Li, and D. R. Deng. 1994. Partial genome sequence analysis of hepatitis E virus isolated by cell culture. Chin. Virol. 9:213–216.
5. Huang, R. T., D. R. Li, J. Wei, X. R. Huang, X. T. Yuan, and X. T. Tian. 1992. Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV). Virology 191:550–558.
6. Huang, R. T., N. Nakazono, K. Ishii, O. Kawamata, R. Kawaguch, and Y. Tsukada. 1995. Hepatitis E virus (87A strain) propagated in A549 cells. J. Med. Virol. 47:299–302.
7. Wei, S. J., P. Walsh, T. Yangbo, H. Q. Dong, and X. L. Cai. 1998. Nucleic acid sequence analysis of the sporadic hepatitis E virus strains in Guangzhou. Chin. J. Microbiol. Immunol. 18:349–361.