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World J Gastroenterol 2005;11(7):970-975
World Journal of Gastroenterology ISSN 1007-9327
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• *VIRAL HEPATITIS* •

Hepatitis G virus genomic RNA is pathogenic to Macaca mulatta

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Received: 2004-06-04 Accepted: 2004-08-05

Abstract

AIM: To explore the pathogenicity and infectivity of hepatitis G virus (HGV) by observing replication and expression of the virus, as well as the serological and histological changes of Macaca mulatta infected with HGV genomic RNA or HGV RNA-positive serum.

METHODS: Full-length HGV cDNA clone (HGVqz) was constructed and proved to be infectious, from which HGV genomic RNA was transcribed *in vitro*. Macaca mulatta BY1 was intra-hepatically inoculated with HGV genomic RNA, HGV RNA-positive serum from BY1 was intravenously inoculated into Macaca mulatta BM1, and then BB1 was infected with serum from BM1. Serum and liver tissue were taken regularly, and checked with RT-PCR, *in situ* hybridization and other immunological, serological, histological assays.

RESULTS: Serum HGV RNA was detectable in all the 3 Macaca mulattas, serological and histological examinations showed the experimental animals had slightly elevated alanine transaminase (ALT) and developed HGV viremia during the infectious period. The histology, immunohistochemistry, and *in situ* hybridization in liver tissues of the inoculated animals demonstrated a very mild hepatitis with HGV antigen expression in cytoplasm of hepatocytes. RT-PCR and quantitative PCR results showed that HGV could replicate in liver.

CONCLUSION: The genomic RNA from full-length HGV cDNA is infectious to the Macaca mulatta and can cause mild hepatitis. HGV RNA-positive serum, from HGV RNA inoculated Macaca mulatta, is infectious to other Macaca mulattas. Macaca mulatta is susceptible to the inoculated HGV, and therefore can be used as an experimental animal model for the studies of HGV infection and pathogenesis.

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Key words: Hepatitis G virus; Genome RNA; Macaca mulatta; Pathogenicity

Ren H, Zhu FL, Cao MM, Wen XY, Zhao P, Qi ZT. Hepatitis G virus genomic RNA is pathogenic to Macaca mulatta. *World J Gastroenterol* 2005; 11(7): 970-975
http://www.wjgnet.com/1007-9327/11/970.asp

INTRODUCTION

Hepatitis G virus (HGV), once named GB virus C (GBV-C), is a positive-sense single-stranded RNA virus whose genetic structure resembles hepatitis C virus and is considered to be a member of Flaviviridae family[1-3]. Early researches support that HGV is associated with post-transfusion hepatitis and other acute or chronic liver- diseases[1,2], but subsequent works question the pathogenicity of HGV[4,5]. Actually, HGV infection is found widely in human population, with frequencies of active or past infection ranging from 5% to 15%[6-8]. HGV infection is frequently persistent and associated with high- level of circulating viremia[9-11]. Until now, many efforts have been made on the expression and replication of HGV *in vitro*, but few researches have focused on the association of HGV with liver diseases[12-16]. Since HGV does not infect routine experimental animals and is difficult to replicate *in vitro*, the pathogenicity, pathogenesis, replication and expression of HGV in host are not clear[17-19]. Although experimental HGV infection with HGV RNA-positive plasma has been reported in chimpanzees and rhesus monkeys, the results are controversial[20-23].

The genome of positive strand RNA virus functions as mRNA, from which all viral proteins necessary for virus propagation are translated. Thus, genomic RNA as well as RNA transcripts from full-length cDNA clones, should be infectious. In fact, it has been proved in cell cultures and animal inoculation studies of HAV and HCV using full-length RNA transcripts[24-26]. In this laboratory, we have constructed a full-length HGV cDNA clone (pHGVqz), which is proved to be infectious *in vitro*[27-29]. pHGVqz is deposited in the GenBank with the accession number of AF081782 and contains 9373 bp in length[30,31]. In the present study, the full-length HGV genome was transcribed from this clone and intra-hepatically injected into the liver of Macaca mulatta to study its pathogenicity[32,33].

MATERIALS AND METHODS

Experimental animals

Three Macaca mulattas (BY1, M, 2 years, 2 kg; BM1, F, 10 m, 1.0 kg; BB1, F, 1 years, 1.5 kg) were used in this study. These

animals were purchased from Chinese Academy of Sciences (Shanghai) and maintained under conditions that met all requirements for use in an approved facility. The animals were not inoculated with any material from animal or human, prior to this study. Serum samples and liver biopsies were taken as negative controls before inoculation. The Macaca mulattas were observed for several months before use and proved to be healthy with normal ALT, negative for HGV RNA, anti-HAV, anti-HBV, anti-HCV and anti-HGV.

In vitro transcription

pHGVqz was linearized with *Xba* I, and transcribed with T7 RNA polymerase, according to the manufacturer's instructions (Riboprobe transcription system, Progema, Madison, WI). The products were digested with 1 u/μg RQ1 DNase at 37 ℃ for 15 min, extracted with phenol/chloroform and precipitated with ethanol. The HGV RNA transcripts were stored at -70 ℃ for use.

Methods of infection

Laparotomy was performed and HGV RNA transcripts (90 μg) were injected into 6 sites of the exposed liver of BY1. One milliliter of HGV RNA-positive serum taken from BY1 at the ninth month post-inoculation was intravenously injected into BM1, and then BB1 was intravenously injected with 1 mL of BM1 serum at the seventh month post-inoculation. Serum samples were collected weekly for the first part of the study after injection, and less frequently thereafter, and monitored for HGV RNA, liver enzymes [alanine aminotransferase (ALT)] and anti-HGV antibodies (HGV IgG ELISA, Sinoclone Ltd, Hongkong). The cut-off value of ALT was 40 IU/L. Liver tissues were taken regularly for the examination of inflammatory changes.

Detection of HGV RNA

Total RNA was extracted from serum or liver tissues using TRIzol LS or TRIzol reagent. RNA pellet was resuspended in 25 μL of RNase-free water. HGV RNA was amplified by RT-PCR with the primer from the 5'-NTR of HGV and with an external primer pair of 5'-ACCGACG-CCTATCTAAGTAGA-3' and 5'-CTTGGAGTCC CTCTCCAAGCC-3', and an internal primer pair of 5'-GACAGGGTTGGTAGGTCGT AAATCC-3' and 5'-AGAGAGACATTGAAGGGCGAC-3'. Reverse transcription was performed at 42 ℃ for 1 h in 20 μL reaction volume using avian myeloblastosis virus reverse transcriptase (Progema) and external antisense primer (for the detection of genomic RNA) or sense primer (for the detection of minus strand RNA). cDNA was amplified with internal primer pair for 35 cycles with denaturation at 94 ℃ for 30 s, primer annealing at 56 ℃ for 30 s, and DNA amplification at 72 ℃ for 45 s. The amplified product was analyzed by electrophoresis through 15 g/L agarose gel containing ethidium bromide followed by UV transillumination. Each set of experiments included a positive control and a negative control.

HGV specific probe tagged with fluorescence (nt 136-112, 5'-FAM-CAGG GTTGGTAGGTCGT-AAATCCCGG-TAMRA-3') was synthesized by Shenyou Biotechnology Company, Shanghai. TaqMan™ PCR detection kit (Perkin–Elmer Applied Biosystems) was used to quantitate HGV genomic RNA and minus RNA in liver tissues of the infected Macaca mulattas.

Histological inspection and immunohistochemical staining

Autopsy tissues from Macaca mulatta were fixed with 100 mL/L formaldehyde and embedded in paraffin routinely. Each paraffin-embedded specimen was sliced into 5-μm thick sections, which were stained with hematoxylin and eosin (HE). The inflammatory changes of liver tissue sections were observed under a light microscope.

Paraffin-embedded liver sections (5 μm) were dewaxed in xylol and re-hydrated through a series of ethanol dilutions and then disposed with H_2O_2 to inactivate endogenous peroxidase, digested for 30 min with 1 g/L tryspin (GIBCO, Gaithersburg, MD). Monoclonal anti-HGV E_2 (kindly provided by Dr. Engel, Roche Diagnostics, Germany), anti-mouse IgG conjugated with horseradish peroxidase (HRP) and the substrate H_2O_2-diaminobenzidine (DAB)/ACE were added to the sections step by step. The normal liver tissue sections were used as negative controls.

In situ hybridization

Full-length HGV cDNA containing plasmid pHGVqz was digested with *Pst* I, and the 3 642 bp fragment containing a 470 bp of HGV 5'-NCR and vector sequence was recovered and self-ligated. In this new constructed plasmid, HGV cDNA fragment was flanked with the T7 (5' to the cDNA insert) and SP6 (3' to the insert) RNA promoters. The recombinant plasmid was digested with *Xba* I or *Eco*R I and the linearized plasmid was transcribed *in vitro* with T7 (*Xba* I linearized plasmid) or SP6 (*Eco*R I linearized plasmid) RNA polymerases in the presence of digoxigenin 11-UTP (DIG DNA labeling and detection kit, Roche Diagnostics, Germany) to generate probes of sense and anti-sense polarity, respectively.

The 10-μm thick frozen liver sections fixed with 40 mL/L paraformaldehyde/0.1 mol/L PBS at 4 ℃ for 1 h, were rinsed with 0.1 mol/L glycine/PBS and 4 mL/L Triton X-100/PBS. After digestion with proteinase K (1 mg/mL) at 37 ℃ for 10 min, the sections were acetylated in 5 mL/L acetic anhydride in 0.1 mol/L triethanolamine (pH 8.0) for 20 min at room temperature. The slices were rinsed in 2×SSC (standard saline citrate) and quickly dehydrated in ethanol. After that, 20 μL of hybridization mixture consisting of 50 mL/L formamide, 100 mL/L dextran sulfate, 1×Denhardt solution, 10 mmol/L *Tris*-HCl pH 8.0, 0.3 mmol/L NaCl, 1 mmol/L EDTA pH 8.0, 10 mmol/L DNA of salmon sperm, and 5 ng of heat-denatured labeled probe were placed on each slice, sealed with rubber solution. The sections and probe were denatured together at 80 ℃ for 10 min. The slices were incubated at 56 ℃ for 16 h. After hybridization, slices were washed for 1 h at 56 ℃ with 3×SSC, digested with RNase A (20 mg/mL), and rinsed in 1.5×SSC and 0.75×SSC at 50 ℃ for 1 h each. The digoxigenin-labeled hybrids were detected with a digoxigenin antibody-alkaline phosphatase conjugate and an enzyme substrate chromogen (nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate) according to the manufacturer's instructions (Dig nucleic

acid detection kit; Roche Diagnostics, Germany). The results were documented under a light microscope. The negative controls included sections without probes and sections with normal liver tissues.

RESULTS

HGV genomic RNA

As seen in Figure 1, the full-length HGV cDNA clone could be transcribed *in vitro*. The majority of HGV genome RNA was about 9 400 nt in length. Compared to the T7 linear control, the integrity of HGV genomic RNA was good and the yield was about 0.8 μg/μL. HGV RNA transcripts were examined by RT-PCR with or without RTase and the expected fragment was about 233 bp. The absence of residual plasmid DNA was confirmed by the negative results in the PCR analysis when the RT step was omitted (data not shown).

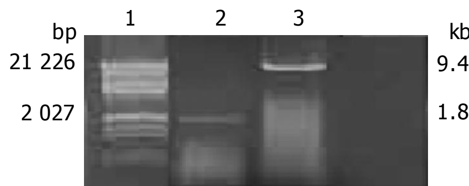

Figure 1 HGV RNA transcripts *in vitro* transcribed from plasmid pHGVqz. Lane 1: molecular standard, lane 2: T7 linear DNA control, lane 3: HGV genomic RNA.

Elevated ALT and positive Anti-HGV

In Macaca mulatta BY1, the ALT level elevated intermittently from wk 4 post-inoculation and kept high for quite a long time lasting 37 wk after inoculation; the peak was 418 IU/L at wk 83 post-inoculation. While in BM1 and BB1, ALT levels elevated 29 and 3 wk after inoculation, respectively (Figure 2). Serum anti-HGV was calculated based on the cut-off values (0.222). Anti-HGV was detectable from wk 39-50 and 64-66 post-inoculation in BY1, at wk 32 and 40-48 post-inoculation in BM1, from wk 6 to 12 post-inoculation in BB1 (Figure 2).

HGV RNA

The expected PCR product was about 233 bp. Positive control and negative samples were set up in each reaction. In Macaca mulatta BY1, serum HGV RNA turned positive from wk 8, kept up for 13 wk and kept intermittently positive thereafter. BY1 was observed for 90 wk then, HGV RNA could also be found in the serum. In BM1, serum HGV RNA became positive from wk 3 to 24, and kept intermittently positive thereafter. While in BB1, serum HGV RNA was only detectable at wk 3 and 4 (Figure 2).

Quantitive PCR showed that the copies of genomic RNA and minus RNA in the liver were 6.24×10^4 and 9.76×10^5/g in BY1, 2.24×10^4 and 1.56×10^6/g in BM1, 1.76×10^4 and 2.88×10^5/g in BB1, respectively.

Histological changes

The histological appearances were normal in all three Macaca mulattas before infection. All of them developed mild inflammatory changes in the liver after the infection (Table 1). Lymphocyte infiltration, focal necrosis, hydropic degeneration and mild proliferation of Kupffer's cells were predominant in the early time and the normal structure of liver lobule was destroyed. When the time of infection was prolonged, the infiltration of lymphocytes and proliferation of fibroblasts became apparent in the portal areas.

Table 1 Pathological finding in hepatocytes of infected Macaca mulattas

Macaca mulatta	Weeks post-inoculation	Histology	Immuno-histochemistry	*In situ* hybridization
BY1	0	Normal	-	-
	18	Chronic mild hepatitis	+	+
	48	Chronic mild hepatitis	+	+
	68	Chronic mild hepatitis	+	+
BM1	0	Normal	-	-
	18	Mild hepatitis	+	+
	32	Chronic mild hepatitis	+	+
BB1	0	Normal	-	-
	18	Mild hepatitis	+	ND

+, positive; -, negative; ND. not done.

Immunohistochemical inspection and in situ hybridization

The immunohistochemistry and *in situ* hybridization in liver tissues were negative before inoculation. HGV E2 protein and HGV mRNA were detectable in hepatocytes of BY1, BM1 and BB1 (Table 1). HGV E2 antigen-positive hepatocytes

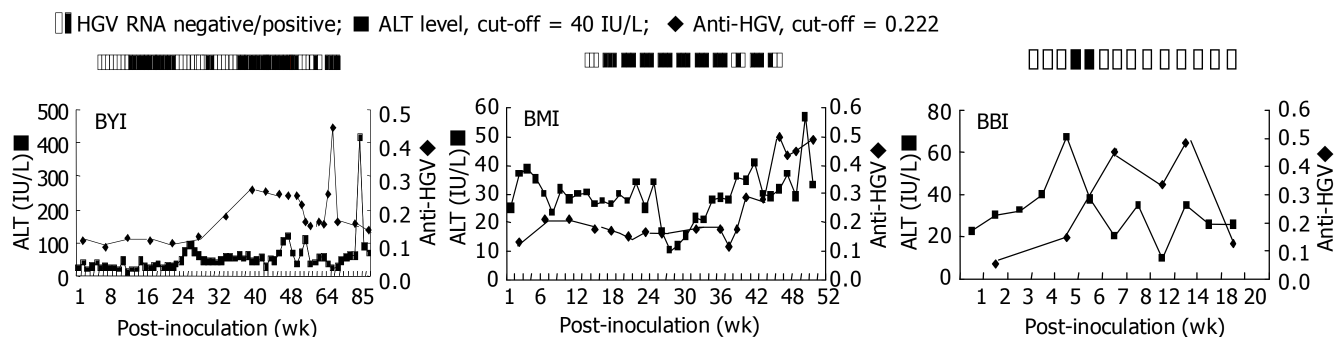

Figure 2 ALT levels, anti-HGV and HGV RNA in inoculated Macaca mulattas.

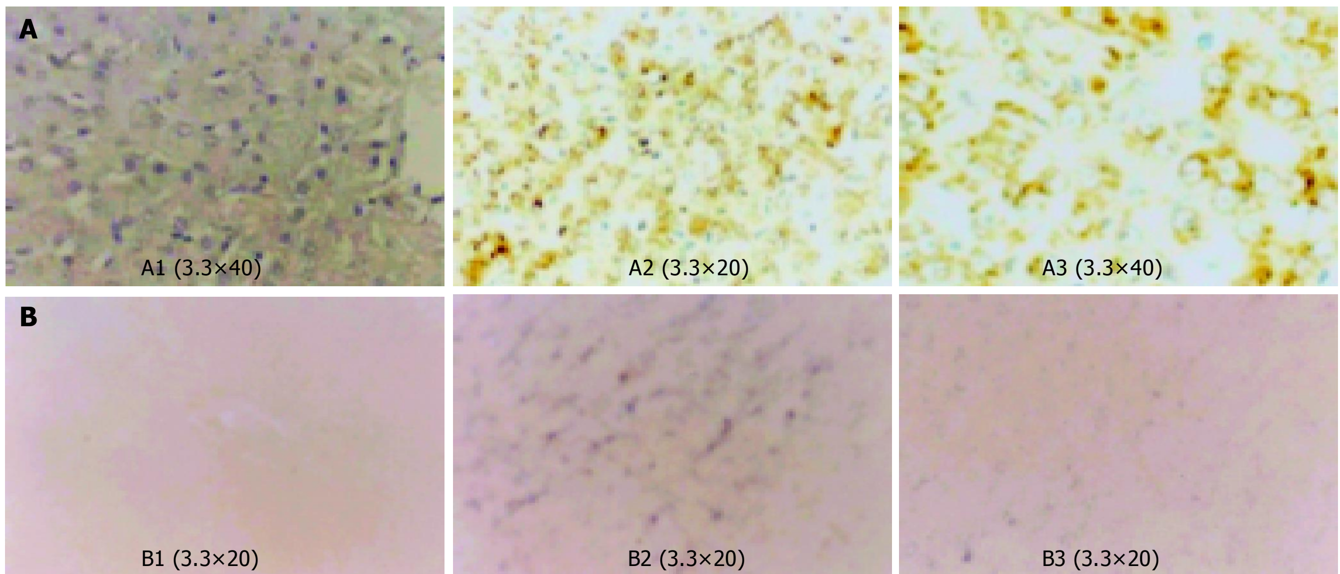

Figure 3 Immunohistochemistry (A) and *in situ* hybridization (B) results of liver from inoculated Macaca mulattas. A1: Normal liver; A2, A3: HGV E2 protein in infected liver; B1: Normal liver; B2, B3: HGV mRNA in infected liver.

were distributed sparsely in liver lobules, mainly in the cytoplasm of hepatocytes. The negative controls in the experiments were normal (Figure 3). The results of immunohistochemistry and *in situ* hybridization were basically coincident.

DISCUSSION

HGV is a newly- identified causative agent of post-transfusion non-A-E hepatitis[1-3]. Although whether HGV could lead to human hepatitis is still controversial, HGV RNA does exist in the sera of both blood donors and various hepatitis patients[6-8]. Besides the epidemiological and clinical studies, some primate animal models have been used to study the pathogenicity of HGV[20-23]. When 2 chimpanzees are inoculated with patient's serum containing 10^8 copies of HGV RNA, viremia occurs at wk 10 and 11 after inoculation but neither of the chimpanzees developed hepatitis[20]. However, elevated ALT, HGV RNA and anti-HGV are detectable[21-23], since the materials used are from patients, which could not exclude the possibility that the undiscovered infectious agents may interfere with the results of experiments. HGV is RNA virus, the genomic RNA serves as template both for viral replication and for protein translation. In order to eliminate other infectious factors from HGV-positive human plasma, we decided to study the pathology and replication of HGV using full-length genomic HGV RNA transcribed from HGV cDNA clone. We constructed a full-length genomic HGV cDNA clone (pHGVqz) in our laboratory, which provided us a good starting material for this study. HGVqz represents the full-length genome of HGV, 9 373 bp in length, and consists of a 5'-noncoding region, an open reading frame, and a 3'-noncoding region. The HGV genomic cDNA was cloned into *Eco*R I and *Xba* I sites of pGEM-3zf (+) vector, and immediately downstream of the T7 promoter, which ensured that the T7 promoter started transcription from the exact 5'-end, stopped at the exact 3'-end of HGV, and produced the authentic HGV RNA transcripts[27,28,30,31]. Before *in vitro* transcription, the plasmid was linearized with *Xba* I. The successful construction of full-length genomic cDNA clone allowed us to avoid the defect of infection with positive plasma. Furthermore, chimpanzee or Macaca mulatta infected with RNA transcripts derived from a single cDNA clone can provide more detailed information on pathogenicity, pathogenesis and evolution of the virus.

In this study, Macaca mulatta BY1 was intra-hepatically injected with HGV RNA transcripts from pHGVqz, Macaca mulatta BM1 was intravenously inoculated with HGV RNA-positive serum collected from BY1 and Macaca mulatta BB1 was infected with serum from BM1. Our data showed that serum HGV RNA of the 3 experimental animals turned positive between the 3rd and 8th wk post-inoculation and existed for quite a long time, suggesting that HGV can not only replicate in Macaca mulatta, but also transmit to normal Macaca mulattas. Quantitive PCR results of both liver and serum (data not shown) showed that HGV RNA decreased with the infection passage, possibly because the virulence reduced during the passage of infection. Intermittently elevated serum ALT level was detectable in all 3 Macaca mulattas without direct association with HGV RNA. Anti-HGV, detectable in the inoculated Macaca mulatta, suggests HGV protein is expressed and immune responses are induced in the Macaca mulattas. Compared to normal liver before inoculation, the 3 Macaca mulattas developed mild hepatitis. HGV E2 protein and mRNA were also detectable using immunohistochemistry and *in situ* hybridization. Serological and histological changes in 3 Macaca mulattas proved that HGV might exist in Macaca mulattas and have potential infectivity.

Although the replication mechanism of HGV is unknown, it is presumed that HGV replicates in the same manner as other positive-stranded RNA flaviviruses. We examined HGV minus strand RNA in the liver to see whether the liver was the replicate site of HGV. In our study, HGV minus RNA was found in the liver of all 3

Macaca mulattas, which was 10-fold more than genomic RNA. Until now, the tissue tropism of HGV is unclear. Some studies reported that HGV minus RNA is found in borne marrow cells and peripheral blood mononuclear cells (PMBC), but not in liver[25,26]. Contradictory results have been reported that HGV minus RNA is detectable in liver and PMBC samples from chronically HGV-infected patients[34-36]. These discrepancies could be partially explained if there are HGV variants with different tissue tropism. Fogeda *et al*[37] reported that both hepatotropic and lymphotropic HGV variants exist in infected hosts. Replication of different tropic variants determines the distribution of serum HGV, and only a fraction of HGV variants present in serum is able to infect and replicate in PBMC *in vitro*. In our study, the expression and replication of HGV were detectable in the livers of experimental animals, suggesting that the HGV RNA genome transcribed *in vitro* has the liver tropism, indicating that HGV RNA genome may be pathogenic to Macaca mulattas and can lead to viremia and inflammatory changes of liver.

In conclusion, HGV genomic RNA is infectious. HGV RNA exists and replicates in Macaca mulattas, and is capable of causing hepatitis in infected Macaca mulattas. Macaca mulatta is susceptible to HGV infection and may be used as an animal model for studying HGV replication and selecting anti-viral drugs.

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