Experimental Infection of Chimpanzees with Hepatitis C Virus of Genotype 5a: Genetic Analysis of the Virus and Generation of a Standardized Challenge Pool

Jens Bukh, Carl L. Appgar, Ronald Engle, Sugantha Govindarajan, Patricia A. Hegerich, Raymond Tellier,* Doris C. Wong, Randy Elkins, and Michael C. Kew

Six major genotypes (genotypes 1–6) of hepatitis C virus (HCV) have been identified. These genetic variants are being transmitted to chimpanzees, the only recognized animal model for the study of HCV. Genotype 5a (strain SA13), a variant found primarily in South Africa, has been transmitted to chimpanzees for the first time. Experimental infection of 2 chimpanzees was characterized by early appearance of viremia and peak virus titers of $10^5$–$10^6$ genome equivalents/mL. The HCV infection was resolved by week 15 after inoculation in 1 chimpanzee and persisted in the other. Both chimpanzees became anti-HCV–positive by week 14 after inoculation. Both chimpanzees developed viral hepatitis. The infectivity titer of a genotype 5a challenge pool prepared from the first passage of HCV in a chimpanzee was $\sim 10^4$ infectious doses/mL. Finally, sequence analysis of strain SA13 confirmed that genotype 5a is genetically distinct from other genotypes of HCV.

Hepatitis C virus (HCV) is an important human pathogen [1]. More than 80% of HCV-infected persons become chronically infected and have increased risk of developing chronic liver disease. Like other Flaviviridae, HCV has a positive-sense single-stranded RNA genome [1]. Its genome consists of the 5′ untranslated region (UTR; $\sim 340$ nt), the open-reading frame (ORF; $\sim 9000$ nt), and the 3′ UTR (200–300 nt). The ORF encodes a polyprotein that is cleaved into at least 10 putative structural and nonstructural proteins.

Genetic analysis of HCV led to the discovery of six major genetic groups (genotypes 1–6) and multiple minor genetic groups (subtypes 1a, 1b, 2a, 2b, etc.) [2, 3]. However, the biologic significance of these genotypes is not clear. Their where it constitutes genetic groups (genotypes 1–6) and multiple minor genetic variants are being transmitted to chimpanzees, the only recognized animal model for the study of HCV. Genotype 5a (strain SA13), a variant found primarily in South Africa, has been transmitted to chimpanzees for the first time. Experimental infection of 2 chimpanzees was characterized by early appearance of viremia and peak virus titers of $10^5$–$10^6$ genome equivalents/mL. The HCV infection was resolved by week 15 after inoculation in 1 chimpanzee and persisted in the other. Both chimpanzees became anti-HCV–positive by week 14 after inoculation. Both chimpanzees developed viral hepatitis. The infectivity titer of a genotype 5a challenge pool prepared from the first passage of HCV in a chimpanzee was $\sim 10^4$ infectious doses/mL. Finally, sequence analysis of strain SA13 confirmed that genotype 5a is genetically distinct from other genotypes of HCV.

Materials and Methods


Experimental animals. Two chimpanzees were used. Serum samples, plasmapheresis units, and liver biopsy samples were taken weekly throughout the first 24 weeks of follow-up and at regular intervals thereafter. Serum was tested for HCV markers (see below), hepatitis B surface antigen, antibodies to hepatitis A, B, D, and E viruses, and serum liver enzyme levels (alanine aminotransferase, $\gamma$ glutamyl transpeptidase, and isocitrate dehydrogenase). Fixed liver tissue was examined for necroinflammatory changes.

Testing for HCV. Sera were tested for anti-HCV with the second-generation ELISA (Abbott, Abbott Park, IL). Sera from the
first-passage chimpanzee were also tested for antibodies to core, E2, NS3, NS3/NS4, and NS5 HCV recombinant proteins with experimental ELISAs done at Abbott.

Total RNA extracted from 100 μL of serum was tested for HCV RNA in reverse transcriptase (RT)–polymerase chain reaction (PCR) by amplification of the 5’ UTR with nested primer pairs [7, 10]. Each experiment included a positive control (10^−6 or 10^−7 dilution of H77 [10]). The HCV genome equivalent (GE) titer was determined by RT-nested PCR on 10-fold serial dilutions of RNA (10^−1 dilution contains the RNA extracted from 100 μL of serum). We defined 1 GE as the number of HCV genomes in the highest dilution that was positive. In HCV RNA–positive samples, the genome titer was determined also by Amplicor HCV Monitor (Roche Diagnostic Systems, Branchburg, NJ) and by Quantiplex HCV RNA bDNA 2.0 (Chiron, Emeryville, CA).

Sequence analysis. The genome of strain SA13 was amplified from serum of chimpanzee 1516 (first passage). Long PCR-RT [11] was used to amplify a segment spanning the 5’ UTR to NS5B, and overlapping regions were next amplified from this long PCR product. A region spanning from NS5B to the conserved region of the 3’ UTR was amplified in RT-nested PCR [12]. Both strands of purified DNA products were sequenced directly to obtain a consensus sequence.

Purified PCR products of the 3’ UTR (amplified with primers H9282F and H3’X45R [12]) were digested with HindIII and XbaI (Promega, Madison, WI), cloned into pGEM-9zf (+) (Promega), and amplified in DH5α competent cells (GIBCO/BRL, Gaithersburg, MD) [12]. Small-scale DNA preparations of 15 clones were sequenced.

Sequence analyses were done using GeneWorks (Oxford Molecular Group, Campbell, CA) and PAUP (version 4.0, created by D. L. Swafford, Laboratory of Molecular Systematics, Smithsonian Institution, Washington, DC), available through the Sequence Analysis Package (version 9.1; Genetics Computer Group, Madison, WI).

Preparation and titration of genotype 5a challenge pool. A first-passage pool (300 mL) was prepared from plasmapheresis units obtained at weeks 1–6 after inoculation from chimpanzee 1516. The units were thawed and pooled on ice, divided into 1.1-mL aliquots, and stored at 15°C. The units were thawed and pooled on ice, divided into 1.1-mL aliquots, and stored at 15°C. The infectious titer of the UTR of other genotype 5a isolates [6]. The variable region of strain SA13 was amplified by reverse transcription in chimpanzee 1547 (figure 1). The 10^−7, 10^−6, and 10^−5 dilutions were noninfectious. However, HCV was transmitted to the chimpanzee by intravenous inoculation of 1 mL of a 10^−5 dilution of the challenge pool, indicating that its infectious titer was ~10^6 chimpanzee infectious doses/mL.

Chimpanzee 1547, inoculated with ~10 GE of HCV, became viremic during week 1 after inoculation (figure 1). Viremia persisted throughout the follow-up period of >1 year, indicating that this second-passage chimpanzee became chronically infected with HCV. The second-generation ELISA for anti-HCV became positive during week 14 after inoculation. After seroconversion, there was a significant decrease in virus titer (from 10^6 to 10^4 GE/mL). The chimpanzee had evidence of hepatitis from week 9 after inoculation onward.

Genetic analysis of strain SA13 of genotype 5a. The consensus sequence of the near-complete genome of HCV from chimpanzee 1516 was determined by direct sequencing of overlapping PCR-amplified genomic regions. The ORF consisted of 9042 nt encoding a single polyprotein of 3014 aa. The consensus sequence could not be determined at five positions with heterogeneity. However, the consensus amino acid sequence was not changed by these differences. The 3’ 246 nt of the 5’ UTR of strain SA13 was identical to those of the 5’ UTR of other genotype 5a isolates [6]. The variable region of the 3’ UTR consisted of 32 nt with two in-frame termination codons. This is the same length as determined for another genotype 5a isolate (FR741) [4]. Two nucleotide differences were found between strains SA13 and FR741 in this region.

The heterogeneity of the 3’ UTR of strain SA13 was analyzed by cloning and sequencing of DNA amplicons obtained by RT-nested PCR. The variable region was highly conserved, with a length of 32 nt in all 15 clones and a total of only two substitutions. The poly U-UC region varied in length (44–127 nt), as well as in composition. The poly U region included one or two A residues in 7 clones, and all 15 clones had one or two G residues in the poly UC region. In all 15 clones, the first 16 nucleotides of the conserved region were identical to the sequence previously determined for genotypes 1, 2, 3, and 4 [13, 14]. However, in strain SA13 of genotype 5a, the sequence 5’-AAATCTTT-3’ was found between the poly UC region and the conserved sequence of the 3’ UTR.

Results

Transmission of HCV genotype 5a to a chimpanzee. Chimpanzee 1516 became infected with strain SA13 [2, 9] after intravenous inoculation of 0.5 mL of serum (containing ~10^6 GE of HCV [in-house RT-PCR, 10^6 GE/mL; bDNA, 10^5.37 GE/mL]) from a South African hepatocellular carcinoma patient (figure 1). The chimpanzee was viremic during weeks 1–14 after inoculation, with titers of 10^4–10^6 GE/mL. Sera collected during weeks 15–26 after inoculation were negative for HCV RNA, indicating that this chimpanzee had an acute resolving infection. The second-generation ELISA for anti-HCV was transiently positive during weeks 14–21 after inoculation. Antibodies to core and NS3 were detected from week 11 after inoculation and throughout follow-up (week 36 after inoculation) with the experimental ELISAs. Antibodies to E2, NS3/NS4, and NS5 were not detected. An IgM anti-core response was detected during weeks 9–13 after inoculation. Evidence of hepatitis (elevated serum liver enzyme values and necroinflammatory changes in liver biopsy samples) was found during weeks 10–21 after inoculation.

Infectivity titration of the HCV genotype 5a challenge pool in a chimpanzee. A pool made from plasmapheresis units collected from chimpanzee 1516 had an HCV titer of ~10^7 GE/mL (in-house RT-PCR, 10^6 GE/mL; Monitor, 10^6.18 GE/mL; bDNA, 10^5.37 GE/mL). Its infectious titer was determined by reverse titration in chimpanzee 1547 (figure 1). The 10^−7, 10^−6, and 10^−5 dilutions were noninfectious. However, HCV was transmitted to the chimpanzee by intravenous inoculation of 1 mL of a 10^−5 dilution of the challenge pool, indicating that its infectious titer was ~10^6 chimpanzee infectious doses/mL.
Figure 1. Course of infection in first-chimpanzee (1516; top) and second-chimpanzee (1547; bottom) passage of SA13 strain of HCV genotype 5a. Chimpanzee 1516 was inoculated intravenously at week 0 with 0.5 mL of human serum containing \( \approx 10^6 \) genome equivalents (GEs) of HCV. Chimpanzee 1547 was inoculated intravenously with \( 10^{-7}, 10^{-6}, \) and \( 10^{-5} \) dilutions, respectively, of genotype 5a (strain SA13) challenge pool (reverse titration). Results of qualitative reverse transcriptase–nested polymerase chain reaction (RT-PCR) for HCV RNA (●, positive; ○, negative), second-generation ELISA for anti-HCV (+, positive; −, negative), and examination of liver biopsy samples for viral hepatitis (necroinflammatory changes graded as 0 [no change], 1+, 2+, 3+, 4+) are shown. Serum levels of alanine aminotransferase (ALT; shaded area) and log_{10} HCV GE titer (in-house RT-PCR, vertical columns; Amplicor HCV Monitor [Roche Diagnostic Systems, Branchburg, NJ], circles; bDNA 2.0, Xs) were plotted against time. HCV titer was below detection limit of bDNA test (postinoculation weeks 5, 9, 11, 12, 14 in chimpanzee 1516 and 1, 2, 14–24 in chimpanzee 1547), as well as Monitor test (weeks 1, 16, 18, 23 in chimpanzee 1547), at several data points (no titer shown).
Figure 2. Phylogenetic analysis of complete open-reading frame sequences and predicted polyproteins of HCV. Sequence of strain SA13 was determined in this study. Genotype designations assigned previously [3, 4, 12, 15] are indicated. Multiple sequence alignments obtained by CLUSTAL W (version 1.6) were manually edited, and positions with deletions or insertions in E2 and NS5A were stripped. Trees were constructed by aid of PAUPSEARCH and PAUPDISPLAY from PAUP software package (version 4.0). Bootstrap 75% majority rule consensus trees (midpoint rooting) were obtained by performing heuristic search (optimality criterion, maximum parsimony; all characters equal weight; 1000 replicates). Total of 8994 nucleic acid (parsimony-informative, 4919) and 2998 amino acid (parsimony-informative, 1275) sites were analyzed. Branch length is drawn in proportion to number of character changes along that branch. Bootstrap values $\geq 75\%$ are indicated. Trees were displayed by aid of TREEVIEW (version 1.5 [16]).

We did multiple sequence alignments of the ORF nucleotide and amino acid sequences of strain SA13 and representative isolates of other genotypes. The sequence identity between SA13 and other genotypes ranged from 67% to 70% at the nucleotide level and 71% to 77% at the amino acid level. The differences observed in the length of the polyprotein of SA13 (3014 aa) and other genotypes (3008–3037 aa) were due to insertions or deletions in E2 and NS5A. Conserved features previously found in other genotypes were also conserved in genotype 5a. For example, the predicted envelope proteins of strain SA13 contained 10 potential N-linked glycosylation sites that are found in all genotypes of HCV.

Recently, the sequence of the complete ORF of another genotype 5a strain (EUH1480) was reported [15]. The ORF of strains SA13 and EUH1480 had the same length, with no insertions or deletions. The amino acid sequences of SA13 at the putative cleavage sites of the nonstructural proteins were identical to those reported for EUH1480 [15]. However, the identity of the ORF sequences of these 2 strains was only 90.3% at the nucleotide level and 92.8% at the amino acid level. The envelope proteins of these 2 genotype 5a strains shared only 83.6% identity. Thus, significant heterogeneity exists among different strains of genotype 5a.

Phylogenetic analyses of the nucleotide sequences (ORF), as well as the deduced amino acid sequences (polyprotein), confirmed that genotype 5a represented a separate major genotype of HCV (figure 2). Six distinct branches, including the branch for genotype 5a, were obtained by distance (not shown) and parsimony analysis of the sequences of isolates representing the recognized variants of HCV. The 2 genotype 5a strains (SA13 and EUH1480) were as closely related as were strains within genotypes 1a, 1b, or 3a.

Discussion

In the present study, we biologically amplified a genotype 5a strain in a chimpanzee and prepared an infectious challenge pool. The HCV genome titer of this pool was $\sim 10^5$ GE/mL and the infectious titer was $\sim 10^6$ chimpanzee infectious doses/mL. This is the first reported experimental infection of chimpanzees with genotype 5 of HCV. The course of the infection was characterized by early appearance of viremia, acute resolving or persistent infection, and development of viral hepatitis.

Although continuous human T cell lines can be infected with HCV, such cultures have proven to be ineffective in propagating this virus. Thus, the chimpanzee model represents the only means of biologically amplifying particular HCV strains. Chimpanzees are susceptible to infection with genotype 1a and 1b isolates, and the course of the infection is reminiscent of infection in humans [5]. The present study extended the susceptibility to genotype 5a and showed that the course of the infections with a genotype 5a strain was indistinguishable from those reported for experimental infections with genotype 1 strains.
The availability of challenge pools of the different HCV genotypes with known infectious titer should be important for future studies of this heterogeneous virus. For example, such pools could constitute the cloning source for constructing infectious cDNA clones of the various genotypes of HCV [12, 17]. Also, these pools could serve as standardized reagents for the quality control of diagnostic assays. Such infectious virus pools should be useful for future testing of the efficacy of vaccine candidates in chimpanzees. The quasispecies nature of HCV is probably important in the pathogenesis of the disease, and therefore the availability of infectious clones [12, 17] would not supersede the use of infectious plasma pools for vaccine studies.

It has been suggested that the final classification of the HCV genotypes should be based on complete genome sequences [3]. The availability of full-length sequences of genotype 5a isolates reported here and in a recent study [15] permits more definitive analyses. Our phylogenetic analyses of complete nucleotide and polyprotein sequences confirm that genotype 5a is a major genotype of HCV. The existence of the six major HCV genotypes (1–6), originally described by analysis of partial genomic sequences, is supported by true phylogenetic analysis of full-length sequences. On the basis of maximum parsimony analysis, the 5a nucleotide sequences were more closely related to members of genotype 3 than to other major genotypes. However, this closer relationship was not confirmed by maximum parsimony analysis of the protein sequences or by distance analysis of nucleotide and deduced amino acid sequences. Isolates from Southeast Asia [4] were proposed to comprise additional major genotypes (7–11). However, on the basis of phylogenetic analysis of full-length sequences, genotypes 10a and 11a should probably be classified within genotypes 3 and 6, respectively (the full-length sequences of genotypes 7, 8, and 9, known to be most closely related to genotypes 6a and 11a [4], have not been published).

In conclusion, chimpanzees were susceptible to infection with the SA13 strain of genotype 5a, which represents one of the six major genotypes of HCV. The course of the infection of this genotype was similar to experimental infection with genotypes 1a and 1b. The infectious challenge pool of genotype 5 should be useful for future studies, including vaccine development.

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References

4. Tokita H, Okamoto H, Iizuka H, et al. Hepatitis C virus variants from Jakarta, Indonesia classifiable into novel genotypes in the second (2e and 2f), tenth (10a), and eleventh (11a) genetic groups. J Gen Virol 1996;77:293–301.