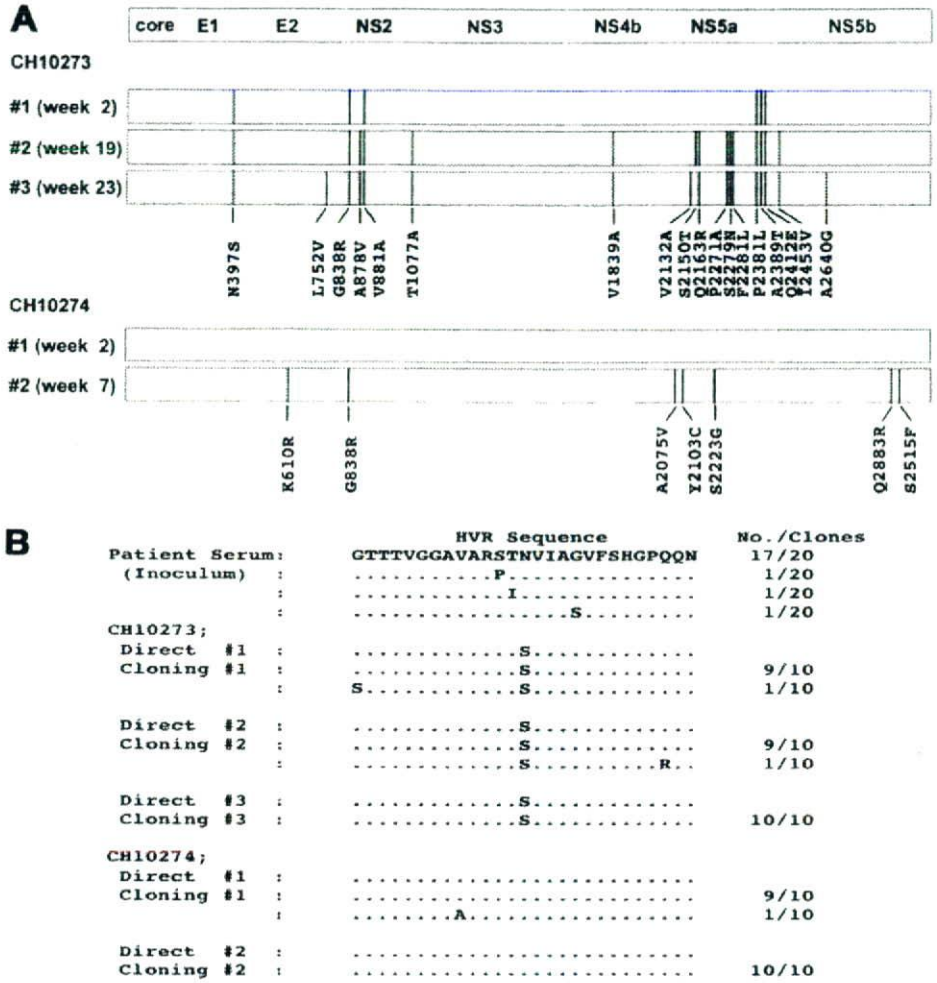

Fig. 2. HCV sequence analyses. (A) Distribution of amino acid substitutions in patient serum–inoculated (CH10273) and JFH-1cc-inoculated (CH10274) chimpanzees. Positions of amino acid substitutions are indicated as vertical bars, and the mutated amino acids are shown at the bottom of each panel. The amino acid numbers correspond to the JFH-1 sequence. (B) HVR-1 populations in patient serum (inoculum) and chimpanzees. HVR-1 sequence in patient serum has been reported previously.[6] HVR-1 sequences determined by direct sequencing (Direct) or cloning and sequencing (Cloning) (10 clones at each time) in each animal are shown. Investigated time points (#1, 2, and 3) are indicated in Fig. 1. Identical amino acids are indicated as dots.

G838R). Viral replication and production of the JFH-1 G838R mutant was compared with that of the WT JFH-1 (JFH-1 WT) by transfecting the *in vitro* transcribed full-length genome RNA into Huh7.5.1 cells. HCV RNA levels in culture media of JFH-1 WT and JFH-1 G838R transfected cells were $2.96 \times 10^6 \pm 1.63 \times 10^5$ and $1.69 \times 10^7 \pm 3.61 \times 10^5$ copies/mL on day 3, and $2.67 \times 10^6 \pm 3.69 \times 10^5$ and $1.14 \times 10^7 \pm 2.23 \times 10^5$ copies/mL on day 5, respectively ($P < 0.05$)(Fig. 3A). In JFH-1 WT and JFH-1 G838R transfected cells, intracellular HCV RNA levels were $1.14 \times 10^8 \pm 1.36 \times 10^7$ and $3.66 \times 10^8 \pm 1.20 \times 10^7$ copies/well on day 3, and $1.67 \times 10^8 \pm 3.94 \times 10^7$ and $2.23 \times 10^8 \pm 1.90 \times 10^7$ copies/well on day 5, respectively ($P < 0.05$) (Fig. 3A). Thus, JFH-1 G838R could produce HCV RNA approximately fivefold higher than the JFH-1 WT in culture media and transfected cells (days 3 and 5, $P < 0.05$).

To confirm this observation, an infection study was also conducted with cell culture–generated viruses. After transfection of JFH1 WT and JFH-1 G838R genome RNA, viruses in culture media were harvested, and FFU of these viruses were titrated. The same titer of JFH1 WT or JFH-1 G838R viruses was inoculated into naïve Huh7.5.1 cells (9×10^2 FFU, multiplicity of infection = 0.003). After infection, HCV RNA titer in culture medium and infected cells was determined. Consistent with the transfection study, HCV RNA levels in culture media of JFH-1 G838R virus–infected cells were threefold to sixfold higher than those of JFH-1 WT virus (days 3 and 5, $P < 0.05$; Fig. 3B). Intracellular HCV RNA level on

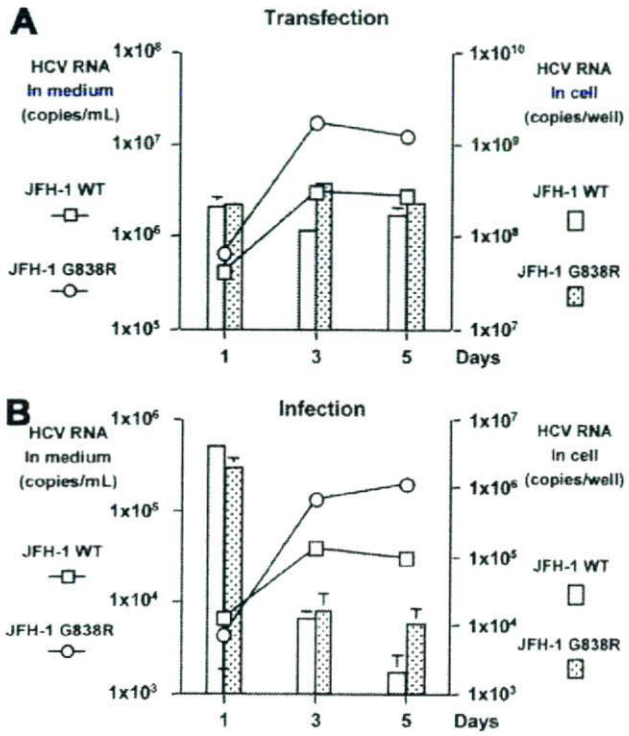

Fig. 3. Comparison of viral replication between JFH-1 WT and JFH-1 G838R in Huh 7.5.1 cells. At various times, HCV RNA was measured in culture media and cells by transfecting the same amount of *in vitro* transcribed full genome RNA (A) and by infecting the same FFU of JFH-1cc at a multiplicity of infection of 0.003 (B). Means of triplicate samples ± standard deviations are shown.

day 5 also appeared to be higher (fivefold) in JFH-1 G838R–infected cells ($P < 0.05$). Based on these data, JFH-1 G838R replicates more efficiently than the WT.

To further investigate the mechanism of this enhanced replication, we reasoned that this mutation could affect any of the viral RNA synthesis, assembly, or secretion steps. To distinguish among these possibilities, we used Huh7-25 cells, a Huh7 cells–derived cell line lacking CD81 expression.[11] This cell line cannot be reinfected by HCV but can support and produce infectious HCV on transfection with the HCV genome, therefore allowing us to address this question without the confounding effect of reinfection. HCV RNA levels of JFH-1 G838R–transfected cells in culture media were eightfold higher on day 1 and threefold higher on day 3 compared with those of JFH-1 WT transfected cells (Fig. 4A, $P < 0.05$). On day 5, the HCV RNA level was still higher in JFH-1 G838R–transfected cells, but the difference was less. The HCV RNA levels of the replication-deficient clone, JFH-1 GND, transfected cells were substantially lower than both NS2 mutant-transfected and WT-transfected cells (Fig. 4A). Similarly, HCV core Ag in culture media showed a significant difference between JFH-1 WT–transfected and JFH-1 G838R–transfected cells (days 1, 3, and 5, $P < 0.05$)(Fig. 4B). HCV core Ag of JFH-1 GND–transfected cells was under the detection limit. In contrast to culture media data, intracellular HCV RNA and core Ag levels in JFH-1 G838R–transfected cells were similar to or slightly lower than those of JFH-1 WT–transfected cells. Therefore, the G838R mutation does not appear to affect RNA replication and probably enhances either the assembly or secretion step.

To distinguish between these two possible effects, we determined the infectivity titer of intracellular viral particles in transfected cells as reported previously.[18] On day 3 after transfection, the intracellular infectivity titer in JFH-1 G838R–transfected cells was approximately four-

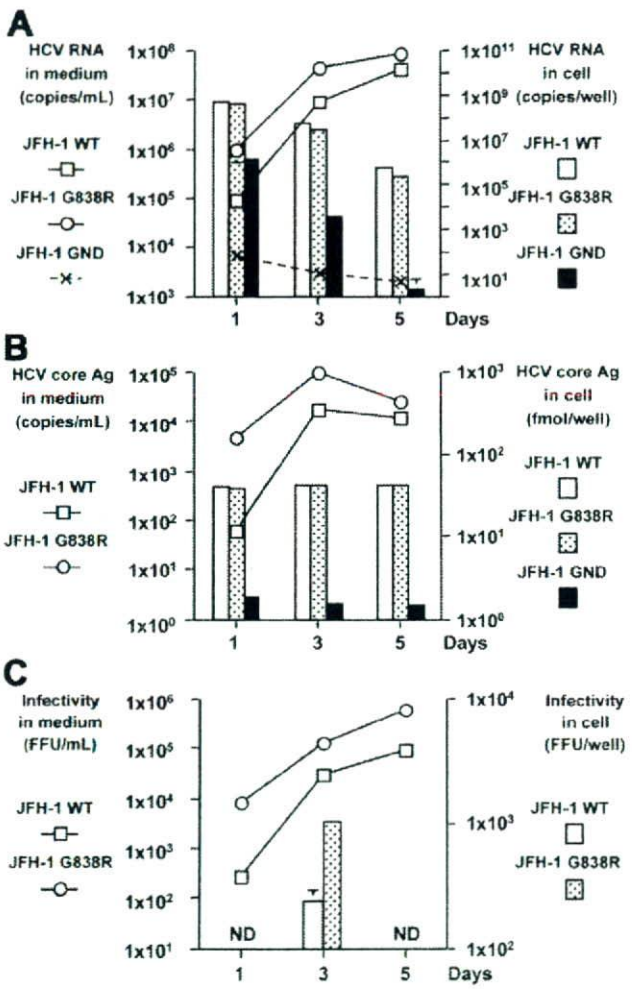

Fig. 4. Comparison of viral replication among JFH-1 WT, JFH-1 G838R, and JFH-1 GND in Huh 7-25 cells. At various times, HCV production was assessed in culture media and cells by transfecting the same amount of *in vitro* transcribed full genome RNA. HCV RNA titer (A), HCV core Ag level (B), and infectivity titers (C) are shown. The data are expressed as means of triplicate samples ± standard deviations. ND, not done.

Table 2. Specific Intectivity and Virus Secretion in Huh7-25 Cells

Clone	Intracellular HCV RNA (copies/well)	Intracellular Intectivity* (FFU/well)	Specific Intracellular Infectivity* (FFU/copies)	Extracellular HCV RNA* (copies/well)	Extracellular Infectivity* (FFU/well)	Specific Exracellular Infectivity (FFU/copies)	Infectious Virus Secretion (extra/intra)
JFH-1 WT	4.40×10^7 ± 1.58×10^7	2.27×10^2 ± 5.17×10^1	1.09×10^{-5} ± 2.58×10^{-6}	1.83×10^7 ± 1.95×10^6	6.17×10^3 ± 9.61×10^2	3.37×10^{-4} ± 1.38×10^{-3}	7.20 ± 2.83
JFH-1 G838R	2.19×10^7 ± 1.11×10^6	9.89×10^2 ± 5.02×10^1	9.05×10^{-5} ± 2.76×10^{-6}	5.14×10^7 ± 3.48×10^6	2.69×10^4 ± 6.96×10^3	5.33×10^{-4} ± 1.83×10^{-4}	6.87 ± 2.07

The data are from day 3 after HCV RNA transfection of the Huh7-25 cells.
*$P < 0.05$ comparing JFH-1 WT and G838R.

fold higher than that in JFH-1 WT–transfected cells ($P < 0.05$, Fig. 4C and Table 2). Moreover, specific intracellular infectivity of JFH-1 G838R–transfected cells was approximately eightfold higher than that in JFH-1 WT–transfected cells ($P < 0.05$, Table 2). Specific infectivity in culture medium was determined as the ratio of infectious virus (FFU) over HCV RNA copies. Specific infectivity of the JFH-1 G838R viruses was not significantly different from that of JFH-1 WT (Table 2). Finally, the rate of secretion was determined by the ratio of extracellular FFU over the intracellular FFU (Table 2), and no difference was observed between JFH-1 WT and G838R-transfected cells. Based on these data, the G838R mutation in JFH-1 enhances the assembly step of HCV.

Discussion

Although HCV-associated fulminant hepatitis is rare, several cases have been reported.[6,19-25] The HCV JFH-1 strain was isolated from one of these cases, and its unique characteristic of robust replication in cell culture might be related to the cause of fulminant hepatitis. Previously, HCV from a patient with fulminant liver failure has been shown to cause severe acute hepatitis with high viremia in a chimpanzee, although its molecular clone could not replicate in culture cells and did not induce severe hepatitis in the chimpanzee.[26,27] In our previous study, JFH-1cc induced a transient and attenuated infection in a chimpanzee.[8] The infection profile was different from the typical course of HCV infection either with patient sera or infectious RNA molecules in chimpanzees.[28-32] Because this observation was unexpected, we reasoned that the lower virulence of this strain *in vivo* might be related to the age of the chimpanzee. The chimpanzee used in the previous study was older (>25 years of age), and older chimpanzees typically do not develop significant disease on HCV infection. Another possible cause was the characteristics of the viral inoculum. JFH-1cc inoculated in the chimpanzee was monotypic because it was generated in culture cells. The original JFH-1 virus replicating in the fulminant hepatitis patient existed as a mixture of various viral species and might induce a different outcome *in vivo*. Thus, to elucidate the pathogenesis and replication capacity of the original JFH-1 strain *in vivo*, the patient serum and the JFH-1cc were inoculated into juvenile chimpanzees (5 years old). However, both chimpanzees showed attenuated infection with low-titer viremia, no ALT elevation, and absence of histological hepatitis during the acute phase of infection. Therefore, the manifestation of fulminant hepatitis of the original patient was likely a result of host factors, with the caveat that humans and chimpanzees might respond differently to HCV infection.

Similar to our previous study, the chimpanzee inoculated with monotypic JFH-1cc showed a short duration of infection and absence of seroconversion. Conversely, the chimpanzee inoculated with the patient serum showed a longer course of infection and developed anti-HCV antibodies. Immunological analysis with T-cell proliferation and IFN-γ ELISpot assays showed that HCV-specific immune responses were similarly induced in both animals and abated with the disappearance of viremia. Consistent with the longer viremia, the chimpanzee inoculated with the patient serum had a longer duration of detectable HCV immune response (Fig. 1). These differences could be explained by the sequence variations of the infecting HCV. In the chimpanzee inoculated with the patient serum, the infecting HCV showed a low sequence complexity but exhibited some sequence diversity already at week 2. The infecting HCV had a sequence alteration in the HVR-1 (N397S), but this sequence alteration could not be found in any of the 20 clones of the inoculum (Fig. 2B).[6] In addition, the NS2 G838R mutation was also not detected by cloning (six clones) and sequencing of the inoculum. Thus, this infecting HCV was probably selected from a minor species in the patient serum. It has been reported that minor clones in human serum were

selected during HCV infection in chimpanzees.[33] The selected clones were in the lighter fraction of the sucrose density gradient of the inoculum, which is devoid of immunoglobulins. Similar selection might have occurred in our study. The dominant clones in the inoculum might not be infectious because of binding to neutralizing antibodies. As a result, the infection-competent minor clone, selected during the infection, became the dominant species. Furthermore, this infecting minor clone could persist longer, although the characteristics of this clone and mechanisms for persistence are still unknown. HCV clones in CH10273 showed several other mutations at 2 weeks postinfection and accumulated additional mutations in E2, NS2, NS3, NS4b, NS5a, and NS5b regions over time (Fig. 2). Some of these regions contain known T-cell epitopes, although the major histocompatibility complex haplotype of this animal is unknown. In this chimpanzee, heterogeneity of the inoculating viruses might have contributed to the emergence of escape mutants from the host immune system, resulting in a prolonged infection. Similar observations have been reported in acute HCV infection in chimpanzees and humans.[34-36]

In HCV strains isolated from these two chimpanzees, one common mutation G838R in the NS2 region was identified. This mutation has not been reported among the adaptive mutations emerged in the JFH-1 virus passaged in cell culture.[37-39] This mutation likely arose *de novo* because one of the chimpanzees was inoculated with a molecular clone, and the week 2 sample did not harbor this mutation. NS2 is a membrane-associated cysteine protease, composed of three transmembrane domains and a protease domain.[40] Although the NS2 region is dispensable for RNA replication, it is essential for production of infectious virus in cultured cells.[41-43] Furthermore, the significance of this region has been shown in the establishment of replication-competent and infection-competent intergenotypical chimeric viruses.[44,45] The identified common mutation G838R was at the end of the first transmembrane domain,[46] and mutations in the transmembrane domains have been shown to improve the yield of infectious virus production in several studies.[45,47] Thus, some advantage of this mutation in HCV replication and production could be expected. This mutation was shown to enhance HCV production in Huh7.5.1 cells. Detailed analysis with CD81-negative Huh7-25 cell demonstrated that viral assembly was affected by this mutation. Production of infectious virus in JFH-1 G838R–transfected cells was eightfold higher than that in the JFH-1 WT–transfected cells. Thus, this mutation enhances the assembly of infectious virus particle in cultured cells, and as a result, increases infectious virus production in the culture medium. This mutation represents the first identified *in vivo* adapted mutation that is not immunologically mediated and probably confers a replication advantage to the virus *in vivo*. This adaptive mutation, unlike the other adaptive mutations reported *in vitro* with poor infectivity *in vivo*, likely results from a highly biologically relevant event in the dynamic interaction between HCV and host. Finally, it is possible that compensatory mutations in other regions of the virus may contribute to the overall biological adaptive response of the virus *in vivo*.

This study demonstrates that the HCV JFH-1 strain either generated in cell culture as a monotypic virus or obtained from patient serum is associated with attenuated infection in chimpanzees; however, the virus can rapidly evolve with adaptive mutations to facilitate propagation of the virus in a susceptible host.

Acknowledgment: The authors thank Charles Rice and Francis Chisari for providing the cell lines and the veterinary staff for technical assistance in the chimpanzee experiment.

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