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Long-Term Elimination of Hepatitis C Virus from Human Hepatocyte Chimeric Mice After Interferon- γ Gene Transfer

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Abstract

Chronic hepatitis C virus (HCV) infection is a leading cause of cirrhosis, liver failure, and hepatocellular carcinoma. Although the combination therapy employing pegylated interferon (IFN)- α and ribavirin is effective, this treatment is effective in only approximately 50% patients with genotype 1 HCV infection. IFN- γ is a potent anti-HCV agent that exhibits its antiviral action through a receptor distinct from that for IFN- α . Therefore, IFN- γ application might provide an alternative approach to IFN- α -based therapies. However, recombinant IFN- γ protein exhibits a poor pharmacokinetic property, that is, a very short half-life. It is our hypothesis that sustained IFN- γ serum concentrations produced by gene transfer could effectively eliminate HCV *in vivo*. We examined the *in vivo* antiviral activity in human hepatocyte chimeric mice infected with genotype 1b HCV at high HCV RNA titers (10^5 – 10^7 copies/ml). The human IFN- γ -expressing plasmid vector pCpG-huIFN γ exhibited prolonged transgene expression in mice compared with the plasmid vector pCMV-huIFN γ . Moreover, the gene transfer of pCpG-huIFN γ eliminated HCV from the liver of the chimeric mice for a sustained period. On the contrary, administration of pCMV-huIFN γ could not eliminate HCV. In conclusion, we found that a single pCpG-huIFN γ injection resulted in long-term elimination of HCV RNA in chimeric mice, providing, for the first time, direct evidence that chronic infection with high titer HCV *in vivo* can be treated by sustained IFN- γ treatment.

Introduction

CHRONIC HEPATITIS C VIRUS (HCV) infection is a leading cause of cirrhosis, liver failure, and hepatocellular carcinoma (Niederau *et al.*, 1998). At present, the standard treatment for chronic HCV patients is a combination of pegylated interferon (IFN)- α and ribavirin (Manns *et al.*, 2001; Fried *et al.*, 2002). However, approximately only 50% of patients with genotype 1 HCV infection and a high viral load showed a sustained viral response, which is associated with resistance to IFN- α (Hofmann *et al.*, 2005; Chayama and Hayes, 2011). More recently, telaprevir, an HCV protease inhibitor, administered in combination with pegylated IFN and ribavirin has led to high rates of sustained virologic response. However, approximately 30% of patients with HCV genotype 1 infection did not respond to the combination treatment (Sherman *et al.*, 2011). Therefore, extensive efforts have been made to develop novel anti-HCV therapies that work via different mechanisms from that of IFN- α .

In vitro cell culture studies using HCV subgenomic replicon systems have demonstrated that IFN- γ (type II IFN) is a potent cytokine with anti-HCV activities (Cheney *et al.*, 2002; Frese *et al.*, 2002; Windisch *et al.*, 2005). The IFN- γ antiviral effects were stronger and more sustained than those of type I IFNs, such as IFN- α and IFN- β (Cheney *et al.*, 2002). Moreover, IFN- γ biological actions are mediated by a unique signal transduction pathway through cell surface receptors that are distinct from those of type I IFNs. Therefore, IFN- γ application could be an alternative therapeutic strategy for chronic HCV infection to overcome the drawbacks associated with present IFN- α -based therapies.

On the basis of the findings regarding IFN- γ anti-HCV activities, the therapeutic efficacy of IFN- γ was evaluated in chronic HCV patients. However, IFN- γ had little or no therapeutic effect in chronic HCV patients (Saez-Royuela *et al.*, 1991; Soza *et al.*, 2005). Although IFN- γ was repeatedly administered at high doses in these studies, the very short *in vivo* half-life of IFN- γ might have hampered its therapeutic

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potential. IFN- γ disappears rapidly from the systemic circulation because it has a half-life ranging from several minutes to several hours after intravenous or intramuscular administration in humans, probably because of its rapid urinary excretion and degradation (Foon *et al.*, 1985; Wills, 1990).

Among the various strategies for manipulating IFN- γ pharmacokinetics, *in vivo* IFN- γ gene transfer could be a useful method if prolonged transgene expression can be achieved. Shin *et al.* (2005) have investigated whether liver-directed IFN- γ gene transfer using viral vectors was effective in reducing serum HCV RNA titers in primates. They did not observe a significant decrease of circulating viral RNA after multiple IFN- γ gene deliveries, and the modest variations in HCV RNA level observed were within the normal fluctuations in the viral load. The IFN- γ mRNA level in the liver after IFN- γ gene transfer was relatively low and rapidly declined with time, probably because of induction of immune responses. We speculated that a limited level and short duration of IFN- γ expression caused by immune responses might be the reason for the poor anti-HCV effect of IFN- γ and that the utilization of a nonviral vector would be effective in overcoming this concern. In our series of studies, we demonstrated that the mouse IFN- γ -expressing plasmid pCpG-mulFN γ produced high serum IFN- γ levels for >80 days after a single administration and produced a significant therapeutic effect in NC/Nga mice, a model for human atopic dermatitis (Hattori *et al.*, 2010). On the basis of these findings, we hypothesized that sustained supplementation of human IFN- γ expressed from the pCpG vector could result in an *in vivo* antiviral effect on genotype 1 HCV.

Repopulation of the liver with human hepatocytes can be induced using immunodeficient urokinase-type plasminogen activator (uPA) mice, and the human hepatocytes in the mice can be used in HCV infection experiments (Mercer *et al.*, 2001). We and other group previously demonstrated that administration of IFN- α and pegylated IFN- α can reduce HCV RNA level in the serum in this model (Inoue *et al.*, 2007; Hiraga *et al.*, 2009; Abe *et al.*, 2011). Here, we investigated the effects of IFN- γ gene transfer on HCV infection using HCV-infected human hepatocyte chimeric mice.

Materials and Methods

Plasmid DNA

The human IFN- γ -expressing plasmid vectors pCpG-huIFN γ and pCMV-huIFN γ (described as phCMVenh/prom-huIFN γ previously) were constructed as described previously (Ando *et al.*, 2012). pCpG-gLuc-encoding *Gaussia* luciferase (GLuc) was constructed by inserting the GLuc cDNA fragment from the pGLuc-Basic vector (New England Biolab, Madison, WI) into pCpG-mcs (InvivoGen, San Diego, CA). The enhanced green fluorescent protein (EGFP)-expressing plasmid vector pEGFP-N1 was purchased from BD Biosciences Clontech (Palo Alto, CA).

Animal treatment

Generation of the immunodeficient uPA^{+/+}/SCID^{+/+} mice and transplantation of human hepatocytes were performed as described previously (PhoenixBio Co., Ltd., Higashihiroshima, Japan) (Tateno *et al.*, 2004). All mice received transplants with frozen human hepatocytes obtained from the same donor. All

animal protocols were carried out in accordance with the guidelines of the local committee for animal experiments (Hiroshima University and Kyoto University). Infection, extraction of serum samples, and euthanization of mice were performed under ether anesthesia. Mouse serum concentrations of human serum albumin (HSA), correlated with the repopulation index (Tateno *et al.*, 2004), were measured using the Human Albumin ELISA Quantitation Kit (Bethyl Laboratories Inc., Montgomery, TX). The chimeric mice with a high replacement rate with human hepatocytes were used in the present study. Eight weeks after hepatocyte transplantation, mice were received an intravenous injection of 10⁵ copies of HCV.

Human serum samples containing a high titer of genotype 1b HCV were obtained from two chronic hepatitis patients (patient-A and patient-B) who had provided written informed consent for their study participation. The study protocol conformed to the ethics guidelines of the 1975 Declaration of Helsinki and was approved by the review committee of Hiroshima University.

In vivo IFN- γ gene transfer in mice and HCV-infected chimeric mice

Gene transfer to mouse liver was performed by the hydrodynamic injection method (Liu *et al.*, 1999), which allows very high transgene expression, especially in the liver. In brief, ICR mice received a rapid injection of 0.11 μ g naked plasmid DNA dissolved in 0.08 ml saline/g body weight into the tail vein within 5 sec. CB17/Icr-Prkd^{scid}/CrjCrlj mice received a rapid injection of 0.22 μ g naked plasmid DNA dissolved in 0.1 ml saline/g body weight into the tail vein within 5 sec. Chimeric mice received 250 μ g naked plasmid DNA dissolved in 0.1 ml saline/g body weight into the tail vein within 5 sec. Transfection efficiency in each chimeric mouse was estimated by measuring the serum level of secretory transgene products, that is, IFN- γ and GLuc, because IFN- γ and GLuc were not detected in untreated chimeric mouse serum and because serum concentration of secretory proteins reflects the transgene expression level in the liver.

Measurement of serum concentrations of IFN- γ , GLuc, and alanine aminotransferase

Mouse sera were collected at indicated times after gene transfer. Human IFN- γ concentrations were determined using a human IFN- γ ELISA kit (Ready-Set-Go! human IFN- γ ELISA; eBioscience, San Diego, CA). To measure serum GLuc activities, serum was mixed with sea pansy luciferase assay buffer (PiccageneDual; Toyo Ink, Tokyo, Japan), and the chemiluminescence was measured in a luminometer. Serum alanine aminotransferase (ALT) level was measured by quantification kit (Transaminase CII test Wako; Wako Pure Chemical, Osaka, Japan).

RNA extraction and amplification

RNA was extracted from serum and liver samples by Sepa Gene RV-R (Sankojunyaku, Tokyo, Japan) and reverse transcribed using a random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace; Toyobo Co., Osaka, Japan). Nested polymerase chain reaction (PCR) and quantitation of HCV by Light Cycler (Roche Diagnostic, Tokyo, Japan) were performed as previously reported

(Tateno *et al.*, 2004; Kimura *et al.*, 2008). The lower detection limits were 10^2 copies/ml for nested PCR and 10^3 copies/ml for real-time PCR.

Fluorescence analysis and histopathological analysis of mouse liver

Mouse livers were excised 1 day after gene transfer with pEGFP-N1, placed in Tissue-Tek OCT embedding compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), and frozen in liquid nitrogen. Frozen liver sections (8 μ m thick) were obtained using a cryostat (Jung CM 3000; Leica Microsystems AG, Wetzlar, Germany). The sections were stained with HSA-specific antibody to visualize human hepatocytes. Liver sections were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). After blocking with 20% fetal bovine serum in PBS, the liver sections were incubated with polyclonal goat antibody against HSA (1:100 dilution). After washing, Alexa Fluor 568 antgoat secondary antibody (1:500 dilution; Molecular Probes, Invitrogen, Carlsbad, CA) was added, and the stained sections were observed using a confocal laser microscope (A1R MP; Nikon Instruments, Tokyo, Japan). Liver samples obtained from pCpG-huIFN γ -injected mice were examined either by hematoxylin–eosin staining or by immunohistochemical staining with anti-HSA antibody as previously described (Hiraga *et al.*, 2007).

Statistical analysis

Differences were evaluated by the Student's *t*-test, and $p < 0.05$ was considered statistically significant. The pharmacokinetic parameters, area under the serum concentration–time curve (AUC), and mean residence time were calculated for each animal by integration to the endpoint of the experiment (Yamaoka *et al.*, 1978).

Results

IFN- γ expressed by pCpG-huIFN γ shows antiviral activity in HCV subgenomic replicon cells

In vitro IFN- γ anti-HCV activity was evaluated using LucNeo#2 cells. Supplementary Fig. S1A (Supplementary Data are available online at www.liebertpub.com/humc) shows the effect of recombinant IFN- γ protein at different concentrations for 1 or 3 days on luciferase activity in LucNeo#2 cells. A dose- and time-dependent inhibitory effect was observed. On the basis of these results, the antiviral activity of IFN- γ expressed after transfection with pCpG-huIFN γ was examined. A significant inhibitory effect was observed after transfection, and a higher inhibition was observed at 3 days (Supplementary Fig. S1B). IFN- γ levels in the culture medium were 2.9×10^5 and 3.3×10^6 pg/ml, corresponding to approximately 700 and 7,800 IU/ml 1 and 3 days after transfection, respectively (Supplementary Fig. S1C). IFN- γ was not detected in the culture supernatant from non-transfected cells.

Hydrodynamic pCpG-huIFN γ and pCMV-huIFN γ injection results in sustained and transient transgene expression of human IFN- γ , respectively

In our previous studies, we demonstrated sustained expression of murine IFN- γ from pCpG-muIFN γ , a CpG-depleted

plasmid vector, in mice after hydrodynamic injection, while transient expression of murine IFN- γ was observed after hydrodynamic injection of pCMV-muIFN γ , a conventional CpG-replete vector (Hattori *et al.*, 2010). To confirm prolonged and transient transgene expression of human IFN- γ from the same types of plasmid vectors, serum IFN- γ concentrations were measured in ICR mice using the hydrodynamic-based method. The serum human IFN- γ concentration was very high in the initial phase and was maintained for up to approximately 240 days after a single hydrodynamic injection (Fig. 1A), while pCMV-huIFN γ failed to show any sustained gene expression. A similar prolonged profile of human IFN- γ was observed in immunodeficient mice for up to 10 days after gene transfer with pCpG-huIFN γ , while the transient profile of serum human IFN- γ concentration was observed after pCMV-huIFN γ injection (Fig. 1B).

Hydrodynamic plasmid DNA injection results in transgene expression in the livers of human hepatocyte chimeric mice

To examine whether *in vivo* gene transfer in the chimeric mice is possible, we conducted an experiment using plasmid DNA encoding GFP to visualize transgene expression in chimeric mouse livers with a high replacement index of human hepatocytes. GFP expression was observed in human hepatocytes, which was confirmed by HSA-specific immunofluorescent staining (Fig. 1C–E). It was found that approximately 1–2% of human hepatocytes were positive for GFP (Supplementary Fig. S2).

A limited antiviral effect can be obtained in chimeric mice infected with genotype 1b HCV after a single injection of pCMV-huIFN γ

To analyze the antiviral effect of IFN- γ gene transfer *in vivo*, chimeric mice with high HCV RNA titers (10^5 – 10^7 copies/ml) 9–12 weeks after HCV inoculation received gene transfer of pCMV-huIFN γ . We measured the HCV RNA titer (upper panel), HSA concentration (middle panel), and IFN- γ concentration (lower panel) in serum after a single injection of plasmid vector (Fig. 2A–D). In a mouse with serum HCV titer of approximately 1×10^6 copies/ml, the HCV RNA level in serum decreased, but not to zero (Fig. 2A). HCV RNA was also detected in the liver of this mouse by nested PCR (Fig. 2E). In other mice with high HCV titer ($> 4 \times 10^6$ copies/ml), serum HCV titers fell slightly 1 week after gene transfer, but the serum HCV titer rebounded later (Fig. 2B and C). In a mouse that showed low serum IFN- γ concentration after the plasmid DNA administration, serum HCV RNA titer did not decrease (Fig. 2D). These results imply that anti-HCV effect observed in the present study is mediated by IFN- γ .

A marked antiviral effect can be obtained in chimeric mice infected with genotype 1b HCV after a single injection of pCpG-huIFN γ

To investigate whether more sustained supplementation of IFN- γ has a more effective antiviral effect in chimeric mice infected with HCV, pCpG-huIFN γ was administered to HCV-infected human liver chimeric mice. As expected, more sustained IFN- γ expression was observed in each chimeric mouse compared with that seen after the administration of

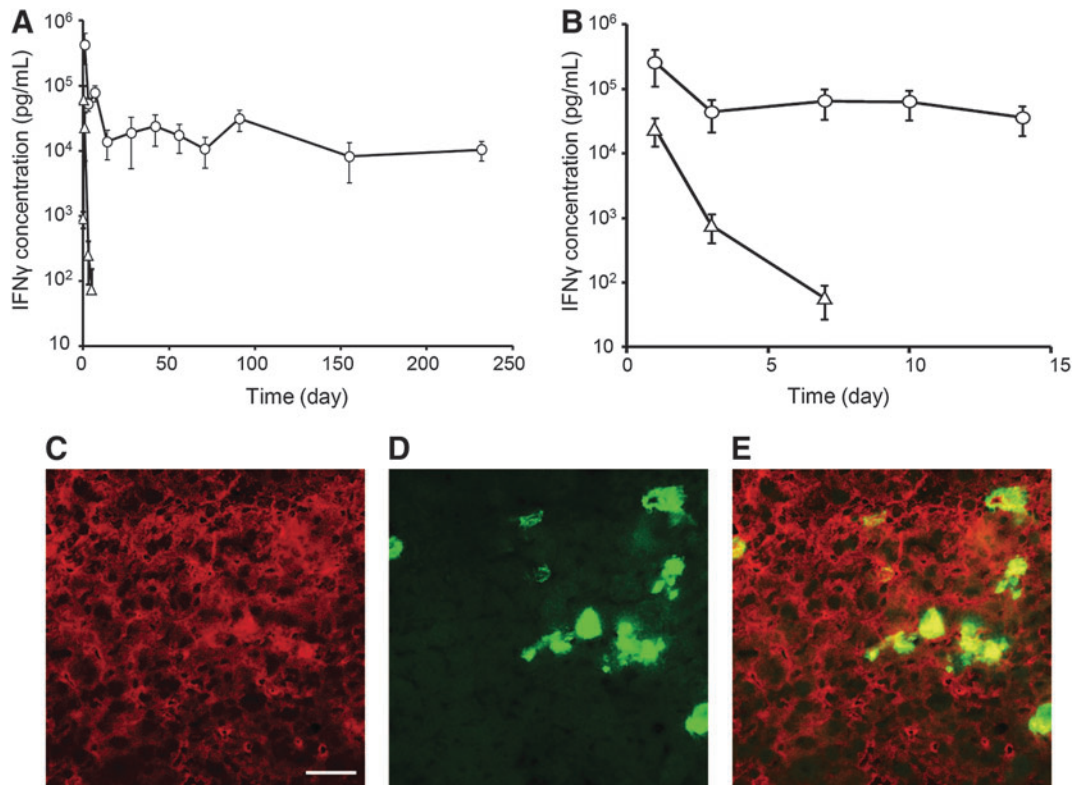


FIG. 1. Prolonged serum concentration of human IFN- γ after hydrodynamic injection of pCpG-huIFN γ and transgene expression in human hepatocytes of the chimeric mice after hydrodynamic administration of plasmid DNA. **(A and B)** Plasmid DNA was hydrodynamically injected into mice. **(A)** Four-week-old male ICR mice (normal mice) received an injection of 0.11 μg of pCpG-huIFN γ (circle) or pCMV-huIFN γ (triangle). **(B)** Six-week-old female CB17/Icr-Prkdc^{scid}/CriCrlj mice (immunodeficient mice) received an injection of 0.22 μg pCpG-huIFN γ (circle) or pCMV-huIFN γ (triangle). Each result represents the mean \pm SD of three mice. **(C–E)** Human hepatocyte chimeric mice were injected with 250 μg of plasmid DNA encoding EGFP. One day after gene transfer, the liver was collected and the liver sections were stained with HSA-specific antibody. Typical images of sections exhibiting red fluorescence **(C; HSA)**, green fluorescence **(D; EGFP)**, and green and red fluorescence **(E; merged image)** are shown. Scale bar = 50 μm . HSA, human serum albumin; IFN, interferon. Color images available online at www.liebertpub.com/humc

pCMV-huIFN- γ , although the absolute IFN- γ levels varied to a great extent. Immediately after pCpG-huIFN γ injection (day 3), HCV RNA levels in the pCpG-huIFN γ -treated mice fell dramatically in 5 of the 6 infected mice (Fig. 3A–E). In a mouse that expressed very low human IFN- γ levels (Fig. 3F), serum HCV titers barely changed after gene transfer with pCpG-huIFN γ . Prolonged undetectable HCV serum levels were observed for more than 7 weeks after gene transfer in all of the surviving mice ($n = 3$) that expressed relatively high human IFN- γ levels. HCV was probably eliminated because no HCV RNA was detected by nested PCR in the livers of the three mice (Fig. 3G). pCpG #4 and pCpG #5 died at 14 and 16 weeks (2 and 4 weeks after IFN- γ gene transfer, respectively) (Fig. 3D and E), although a significant reduction in HCV titers after IFN- γ gene transfer was observed in both mice. These results are summarized in Table 1.

The HCV RNA level in serum was not affected in chimeric mice infected with genotype 1b HCV after a single injection of GLuc-expressing plasmid DNA

Figure 4 shows the results after a hydrodynamic injection of a control vector, pCpG-gLuc, that expresses a reporter protein without any biological activity. Significant GLuc expression was observed in all HCV-infected chimeric mice after

in vivo gene transfer with pCpG-gLuc. Serum HCV RNA levels did not change in the chimeric mice injected with the control vector, which clearly indicates that the antiviral effect observed in the pCpG-huIFN γ -treated mice was caused by IFN- γ expression and not by hydrodynamic plasmid DNA injection. After hydrodynamic plasmid DNA injection, the HSA levels were hardly affected in any of the mice irrespective of the transgenes encoded by plasmid DNAs.

No apparent damage was observed in hepatocytes of chimeric mice after sustained gene expression of IFN- γ

To evaluate hepatic toxicity induced by IFN- γ , serum ALT was measured before and after hydrodynamic injection of pCpG-huIFN γ . As shown in Fig. 5, serum ALT activity increased at 3–7 days after the administration, but it returned to the level comparable to that before the administration later. As it was found that serum ALT increased at 3 days after the administration of pCpG-huIFN γ , livers were collected at 3 days after the plasmid DNA administration. The liver sections were subjected to hematoxylin eosin staining or to HSA-specific immunohistochemical staining. No obvious damage was observed in the chimeric mouse liver compared with the HCV-infected mouse left untreated (Fig. 6A–D).

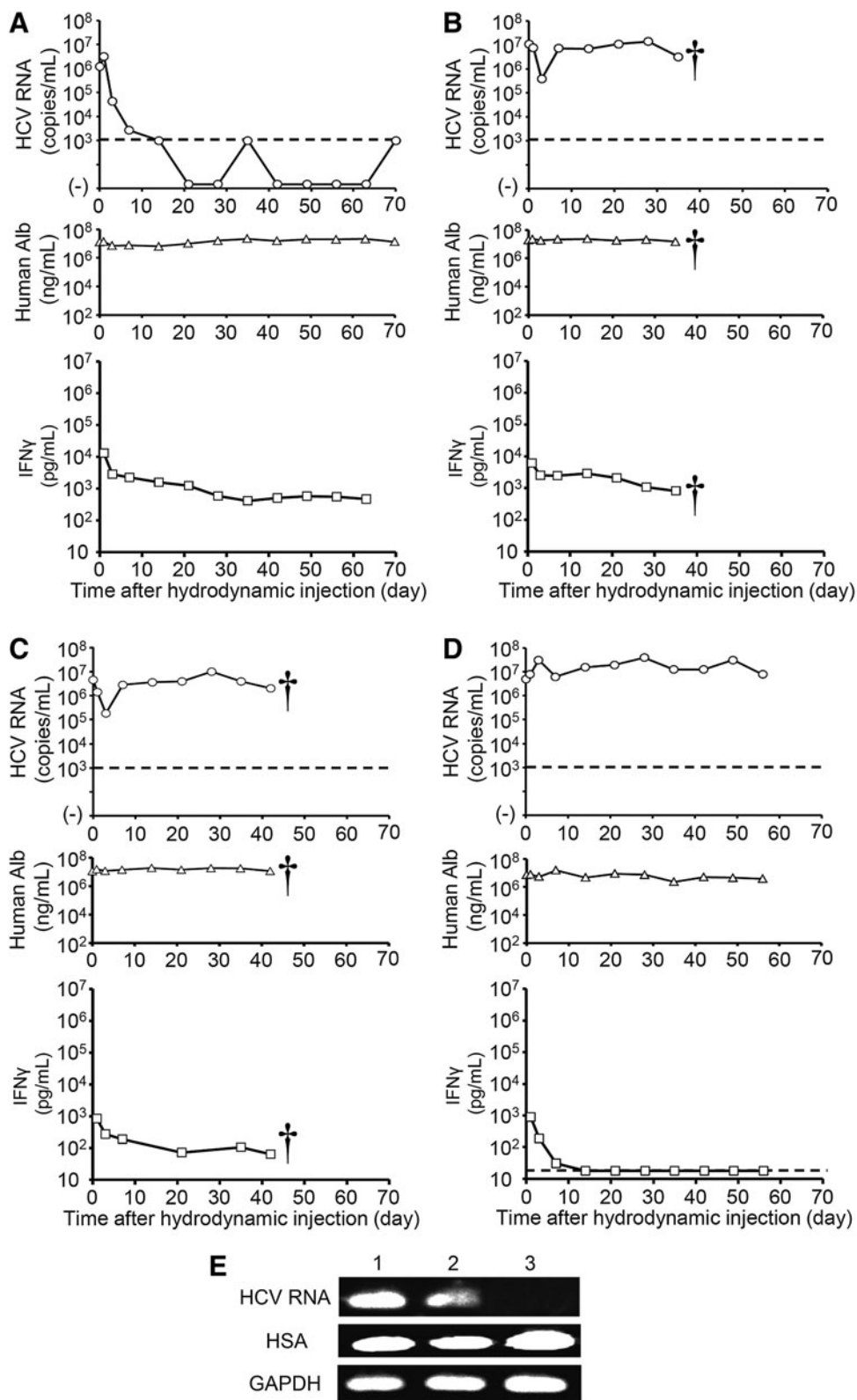


FIG. 2. Effects of intravenous injection of pCMV-huIFN γ in HCV 1b-infected mice. (A–D) Mice that had been injected with HCV-positive human serum from patient-A received 250 μ g of pCMV-huIFN γ . Panels (A–D) correspond to pCMV #1–4, respectively. Serum concentration of HCV RNA copy number (O), HSA (Δ) and IFN- γ (\square) is shown. The horizontal dashed line represents the detection limit (10^3 copies/ml for HCV RNA and 18 pg/ml for IFN- γ). “(-)” on the vertical axis indicates negative for HCV by nested PCR. Dagger (†) indicates the time at which the mice died. (E) Nested PCR in pCMV-huIFN γ -treated mouse livers (lane 2: pCMV #1). Mouse livers with (lane 1) or without (lane 3) HCV infection were also analyzed as controls. HCV, hepatitis C virus; PCR, polymerase chain reaction.

To further evaluate a possible toxic effect on the liver induced by sustained IFN- γ expression in the liver, livers were collected from the pCpG-huIFN γ -treated chimeric mice or pCpG-gLuc-treated chimeric mice at the end of the experiments. As shown in Fig. 6E–H, no obvious damage was ob-

served in pCpG-huIFN γ -treated chimeric mouse liver compared with the liver of pCpG-gLuc-treated chimeric mice. Moreover, IFN- γ expression resulted in no apparent reduction of mice serum HSA levels (Fig. 3), suggesting that sustained IFN- γ exposure would not induce severe toxicity in the liver.

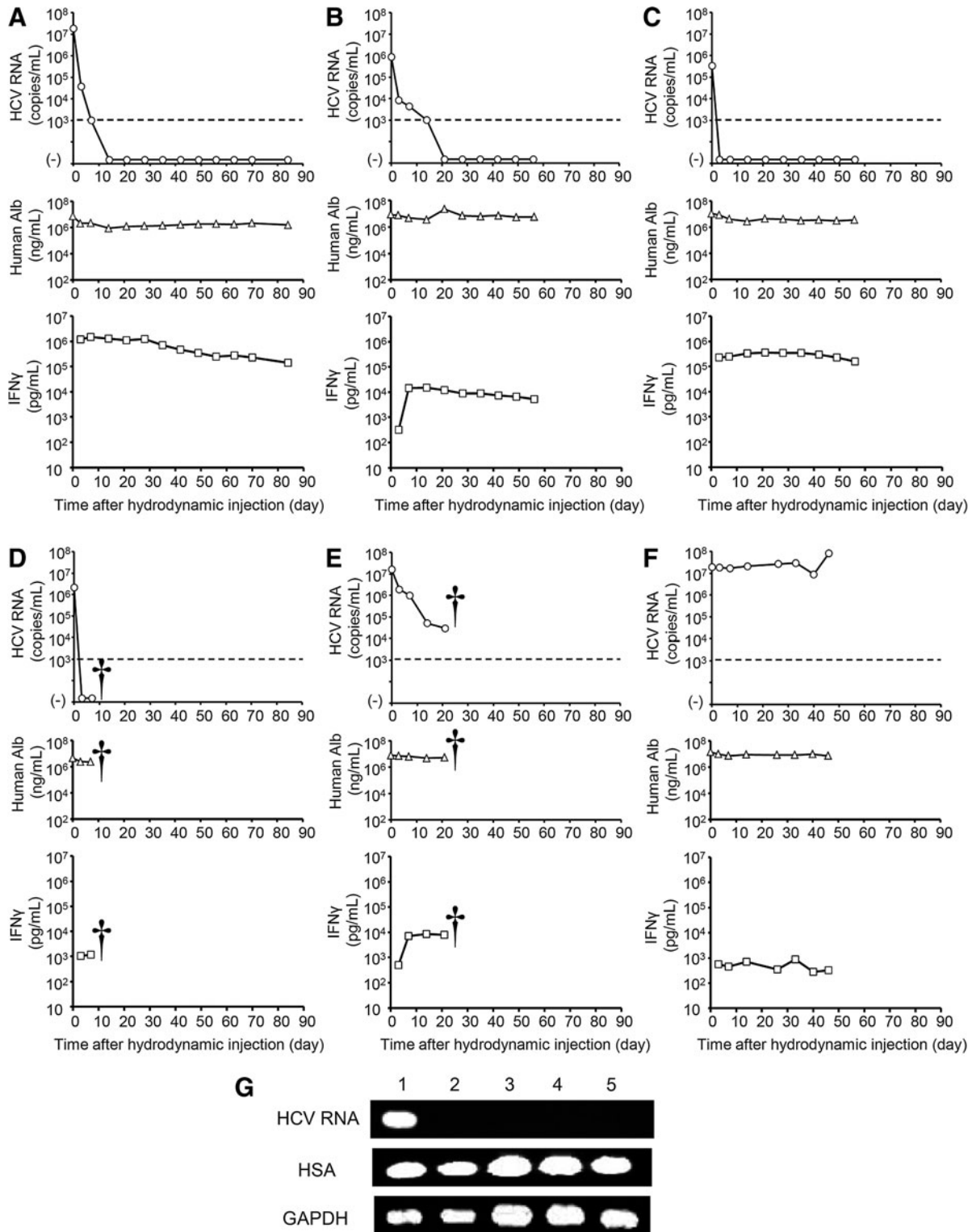


FIG. 3. Effects of intravenous injection of pCpG-huIFN γ in HCV 1b-infected mice. (**A–F**) Mice that had been injected with HCV-positive human serum from either patient-A or patient-B received 250 μ g of pCpG-huIFN γ . Panels (**A–F**) correspond to pCpG #1–6, respectively. Serum concentration of HCV RNA copy number (\circ), HSA (Δ) and IFN- γ (\square) is shown. The horizontal dashed line represents the detection limit (10^3 copies/mL). “(-)” on the vertical axis indicates negative for HCV by nested PCR. Dagger (\dagger) indicates the time at which the mice died. (**G**) Nested PCR in pCpG-huIFN γ -treated mouse livers (lanes 2–4: pCpG #1–3). Mice livers with (lane 1) or without (lane 5) HCV infection were also analyzed as controls.

TABLE 1. SUMMARY OF INTERFERON- γ PHARMACOKINETIC PARAMETERS AND ANTI-HEPATITIS C VIRUS EFFECTS AFTER ADMINISTRATION OF INTERFERON- γ -EXPRESSING PLASMID VECTORS

Mouse	Source	HCV RNA at day 0 (copies/ml)	Human Alb at day 0 (ng/ml)	IFN- γ pharmacokinetic parameters		HCV RNA detection
				AUC (pg·day/ml)	Mean residence time (day)	
pCMV #1	Patient-A	1.2×10^6	1.3×10^7	8.0×10^4	17	±
pCMV #2 ^a	Patient-A	1.1×10^7	2.0×10^7	7.5×10^4	14	+
pCMV #3 ^a	Patient-A	4.5×10^6	1.1×10^7	6.1×10^3	14	+
pCMV #4	Patient-A	5.0×10^6	7.4×10^6	2.0×10^3	1.8	+
pCpG #1	Patient-A	1.8×10^7	7.6×10^6	5.6×10^7	27	–
pCpG #2	Patient-A	8.8×10^5	9.0×10^6	5.1×10^5	25	–
pCpG #3	Patient-B	3.3×10^5	1.1×10^7	1.6×10^7	27	–
pCpG #4 ^a	Patient-A	2.1×10^6	4.6×10^6	6.2×10^3	4.6	–
pCpG #5 ^a	Patient-A	1.6×10^7	7.8×10^6	1.3×10^5	13	±
pCpG #6	Patient-A	1.9×10^7	1.5×10^7	1.3×10^4	13	+

pCMV #1–4 correspond to Fig. 2A–D, and pCpG #1–6 correspond to Fig. 3A–F, respectively.

^aMouse died before the end of the experiments.

AUC, area under the serum concentration–time curve; HCV, hepatitis C virus; IFN, interferon.

Discussion

Human hepatocyte chimeric mice are useful model animals and have been used in variety of basic experiments involving pharmacological and toxicological investigations associated with drug metabolism by cytochrome P450s (Katoh *et al.*, 2008; Yoshizato and Tateno, 2009; Sanoh *et al.*, 2012) and mechanistic studies of viral infections (Sainz Jr. *et al.*, 2012), as well as in therapeutic approaches to HCV infection (Hiraga *et al.*, 2007; Meuleman *et al.*, 2012). In the present study, we utilized human hepatocyte chimeric mice to test our hypothesis that a sustained IFN- γ supply could be a promising therapeutic option for the treatment of chronic HCV infection.

The present study has demonstrated that a single gene transfer of IFN- γ can result in a significant antiviral effect on human HCV in human hepatocyte chimeric mice with high HCV RNA titers (10^5 – 10^7 copies/ml). To our knowledge, this is the first report that clearly demonstrates the *in vivo* effectiveness of IFN- γ on a high titer HCV infection model. It was apparent that the anti-HCV effect obtained by pCpG-huIFN γ in this study was significantly greater than that of IFN- γ recombinant protein in the same chimeric mouse model under a similar high HCV burden (Ohira *et al.*, 2009). Anti-HCV effects were observed after repeated administration of recombinant human IFN- γ at high doses (1×10^5 IU on the first day and thereafter 2×10^4 IU/day for 13 days) when the serum HCV RNA titers were $< 10^3$ copies/ml. However, when the titers increased ($> 10^3$ copies/ml), the preventive effects of recombinant IFN- γ on HCV infection were no longer observed. In the present study, HCV rebound was not observed for up to 8 weeks after a single gene transfer of pCpG-huIFN γ (Fig. 3), and the sustained HCV RNA elimination from the liver was confirmed by nested PCR. As hydrodynamic injection of pCpG-huIFN γ results in IFN- γ transgene expression in the liver, high local concentration of IFN- γ in the liver may be a reason for the strong antiviral effect. Further study is required to determine whether IFN- γ gene delivery into other organs than the liver shows a significant anti-HCV effect.

In the present study, we also tested the antiviral effect of transient IFN- γ expression by using pCMV-huIFN γ . Serum

concentration of IFN- γ in the chimeric mice injected with pCMV-huIFN γ was lower and less sustained than that in the mice injected with pCpG-huIFN γ , although IFN- γ expression from pCMV-huIFN γ was more persistent in the chimeric mice compared with that in ICR mice (Fig. 1A). As far as antiviral effect was concerned, only a marginal or no effect was obtained in mice that received pCMV-huIFN γ . It is noteworthy that HCV rebound was observed in the pCMV-huIFN γ -treated mice with reduced HCV titer in serum, suggesting that long-term IFN- γ supplementation is more favorable for preventing HCV rebound.

We have summarized the IFN- γ pharmacokinetics and anti-HCV effects after IFN- γ gene transfer in Table 1. In the case of a high virus titer ($> 4 \times 10^6$ copies/ml serum), a strong antiviral effect was observed in a mouse that showed a high IFN- γ AUC (5.6×10^7 pg·day/ml; pCpG #1) and in a mouse exhibiting a moderate IFN- γ AUC (1.3×10^5 pg·day/ml; pCpG #5). However, little or no antiviral effect was observed in mice with a high virus titer ($> 4 \times 10^6$ copies/ml serum) and a small IFN- γ AUC ($< 10^5$ pg·day/ml; pCMV #2–4 and pCpG #6). When the virus titer was relatively low ($< 4 \times 10^6$ copies/ml serum), viral clearance or a strong antiviral effect was observed irrespective of the IFN- γ AUC (pCMV #1, pCpG #2–4). These results indicate that a higher IFN- γ concentration is required to obtain anti-HCV effects in HCV infection with a high titer, while a relatively low IFN- γ concentration is enough to obtain anti-HCV effects in HCV infection with a low titer. In addition to the duration of IFN- γ expression, a transient high concentration of IFN- γ may contribute to a potent antiviral effect, as shown in pCMV #1. We also found that the rate of HCV reduction after IFN- γ was different among mice; that is, the HCV titer in pCpG #3 and #4 decreased more rapidly than that in the other responding mice. Although the detailed mechanism is not clear, there was a tendency that the higher the HCV titer, the slower the response to IFN- γ gene expression, indicating that HCV titer is also an important factor in determining the response of IFN- γ treatment.

SCID mice without functional T and B lymphocytes possess normal NK cell function and elevated hemolytic complement activity and retain their innate immune response

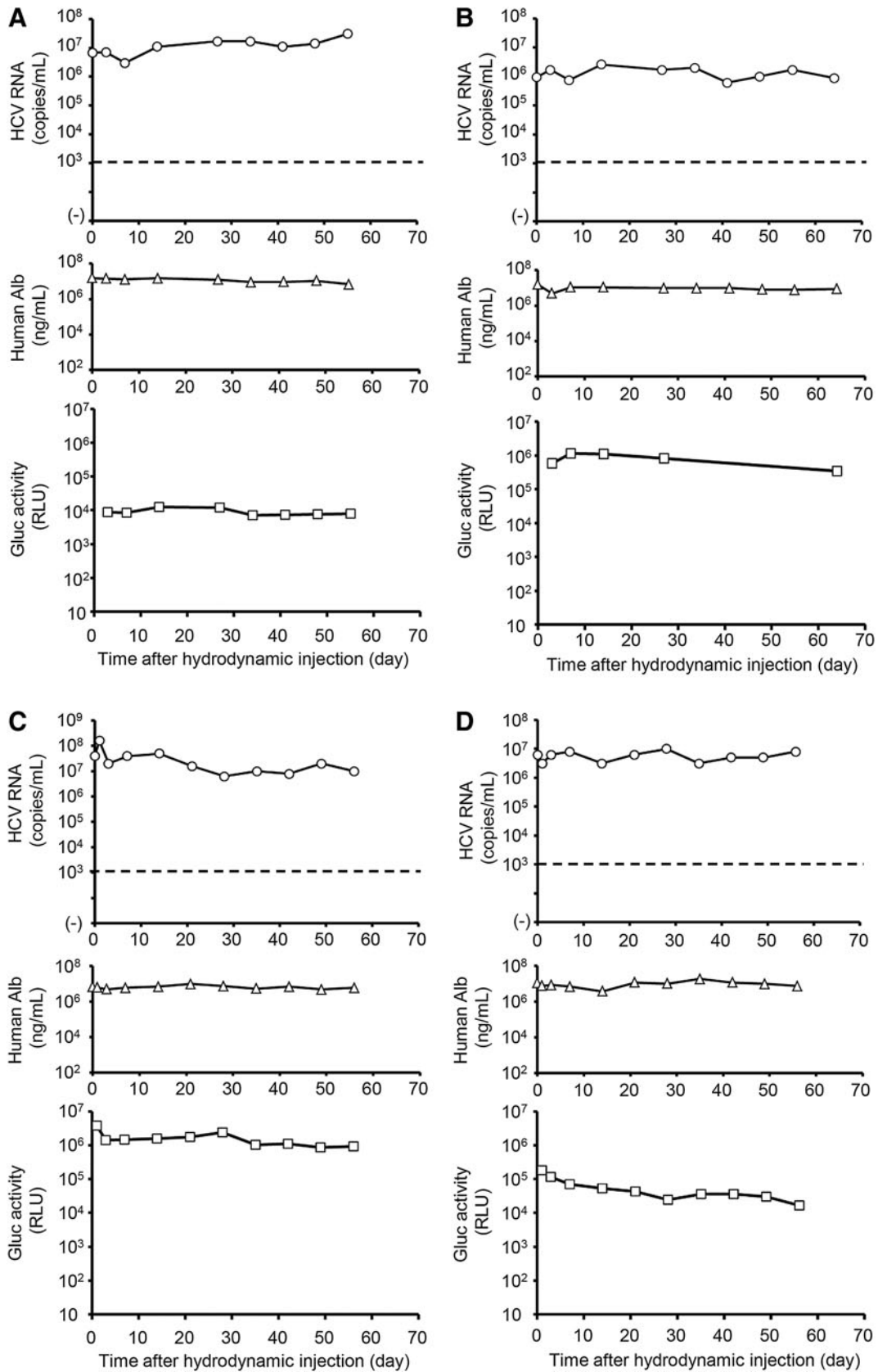


FIG. 4. Effects of pCpG-gLuc (control plasmid DNA) injection in HCV 1b-infected mice. Mice were injected intravenously with HCV-positive serum from patient-A. HCV-infected chimeric mice were injected with 250 μ g of pCpG-gLuc. Data for individual mice are shown in panels (A–D). Serum concentration of HCV RNA copy number (○), HSA (Δ) and gLuc activity (\square) is shown. The horizontal dashed line represents the detection limit (10^3 copies/mL). “(-)” on the vertical axis indicates negative for HCV by nested PCR.

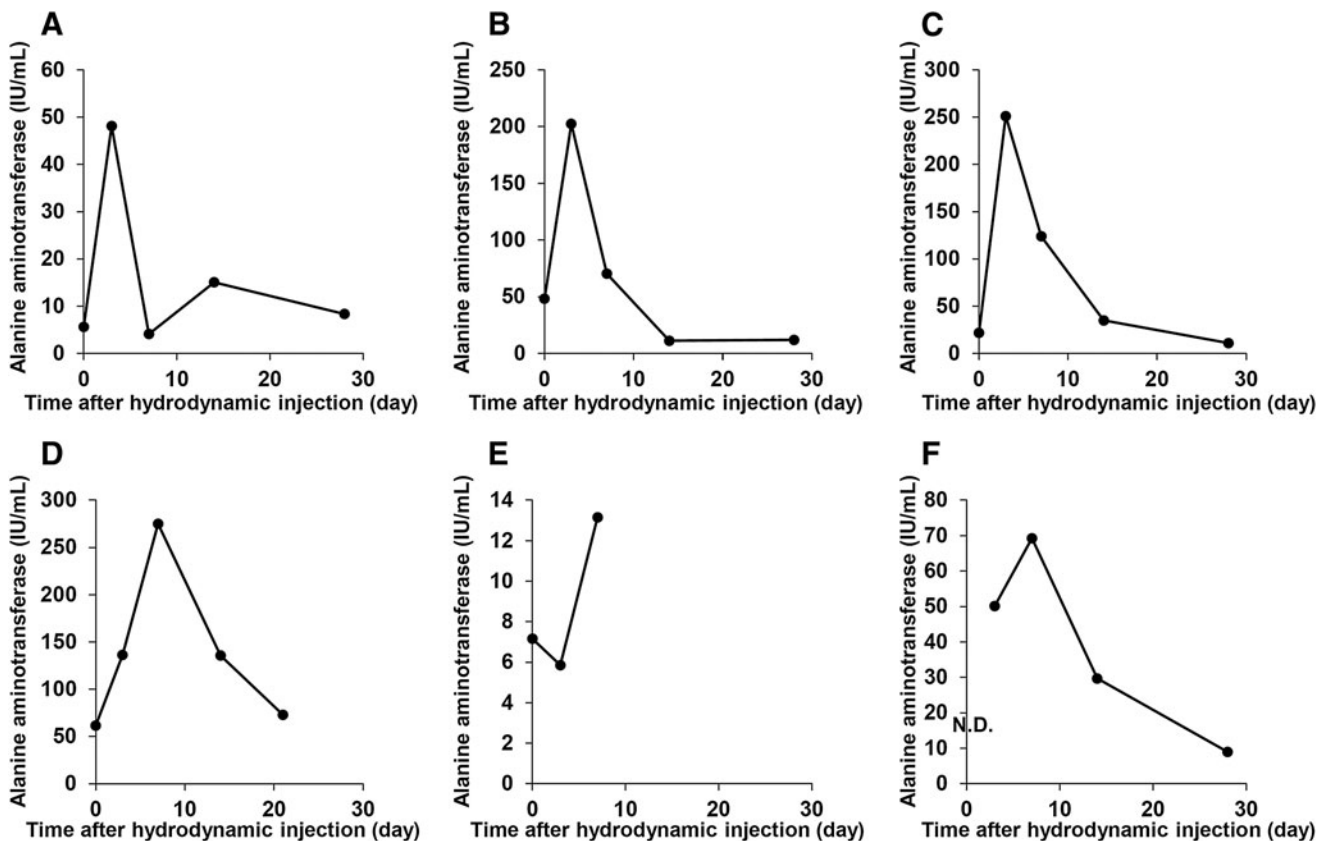


FIG. 5. Serum ALT levels in chimeric mice receiving hydrodynamic injections. Serum ALT levels in chimeric mice receiving pCpG-huIFN γ were measured. Panels (A–F) correspond to pCpG #1–6, respectively. N.D. indicates that serum ALT activity was not determined because of the exhaustion of serum sample. ALT, alanine aminotransferase.

(Shultz *et al.*, 1995). It is possible that introduction of IFN- γ produced anti-HCV effects by activation of resident NK cells. In the present study, flow cytometry analysis showed that activated NK cells were present in the liver 3 days after pCpG-huIFN γ treatment (Supplementary Fig. S3). However, the NK cell populations in the pCpG-huIFN γ -treated mouse livers were similar to those in pCpG-gLuc-treated mice. These results indicate that the anti-HCV effect of IFN- γ in this study seems to be caused by direct inhibition of viral replication.

During the observation period in the present study, two out of six mice injected with pCpG-huIFN γ died and two mice died after pCMV-huIFN γ injection. This was not caused by IFN- γ expression because the level of expressed IFN- γ was relatively low in the dead mice compared with that of survived mice. Vanwolleghem *et al.* (2010) have reported that chimeric mice are weak, and approximately 50% of mice die spontaneously 6 weeks after transplantation. In fact, we also observed some chimeric mice receiving hydrodynamic injection without plasmid DNA spontaneously died in preliminary experiments. Therefore, these four mice were highly unlikely to have died from hydrodynamic injection of IFN- γ -expressing plasmid DNA.

As a high level of IFN- γ might produce toxicity, we measured the serum level of ALT in the chimeric mice. On day 3 after injection of pCpG-huIFN γ , transient increase in the serum level of ALT was observed in the mice that expressed a relatively high level of IFN- γ (Fig. 5). However, 2

weeks after the gene transfer, the serum level of ALT returned to the level comparable with that obtained before the gene transfer. In the case of pCpG-gLuc injection, no significant change was observed (data not shown). Although these results suggest that a high level of IFN- γ expression in the liver may cause slight hepatotoxicity for a short period, which was not detected by histological analysis (Fig. 6C and D), the effect would be transient and limited. The serum level of HSA, an indicator of human hepatocyte number in chimeric mouse livers, did not markedly change after gene transfer of IFN- γ , and a sustained and constant transgene expression from pCpG vectors from the hepatocytes in the chimeric mice was also observed, implying that transgene-expressing hepatocytes were not damaged by IFN- γ . Histological analysis by hematoxylin–eosin staining of the liver sections also suggested that there was no significant damage in human hepatocytes even after long-term exposure to the human cytokine, IFN- γ (Fig. 6G and H). Taken together, these results suggest that sustained IFN- γ treatment would be tolerable in terms of hepatic toxicity induced by IFN- γ .

In the development of IFN- γ gene therapy as the treatment of patients with chronic HCV infection, the method of gene delivery presents a major challenge. In the present study, we found that human hepatocytes in the mouse liver can be transfected by hydrodynamic injection with a large variation in the transfection efficiency among the mice tested. Although the reasons for this variable transfection efficiency in human liver chimeric mice are not clear, some speculations

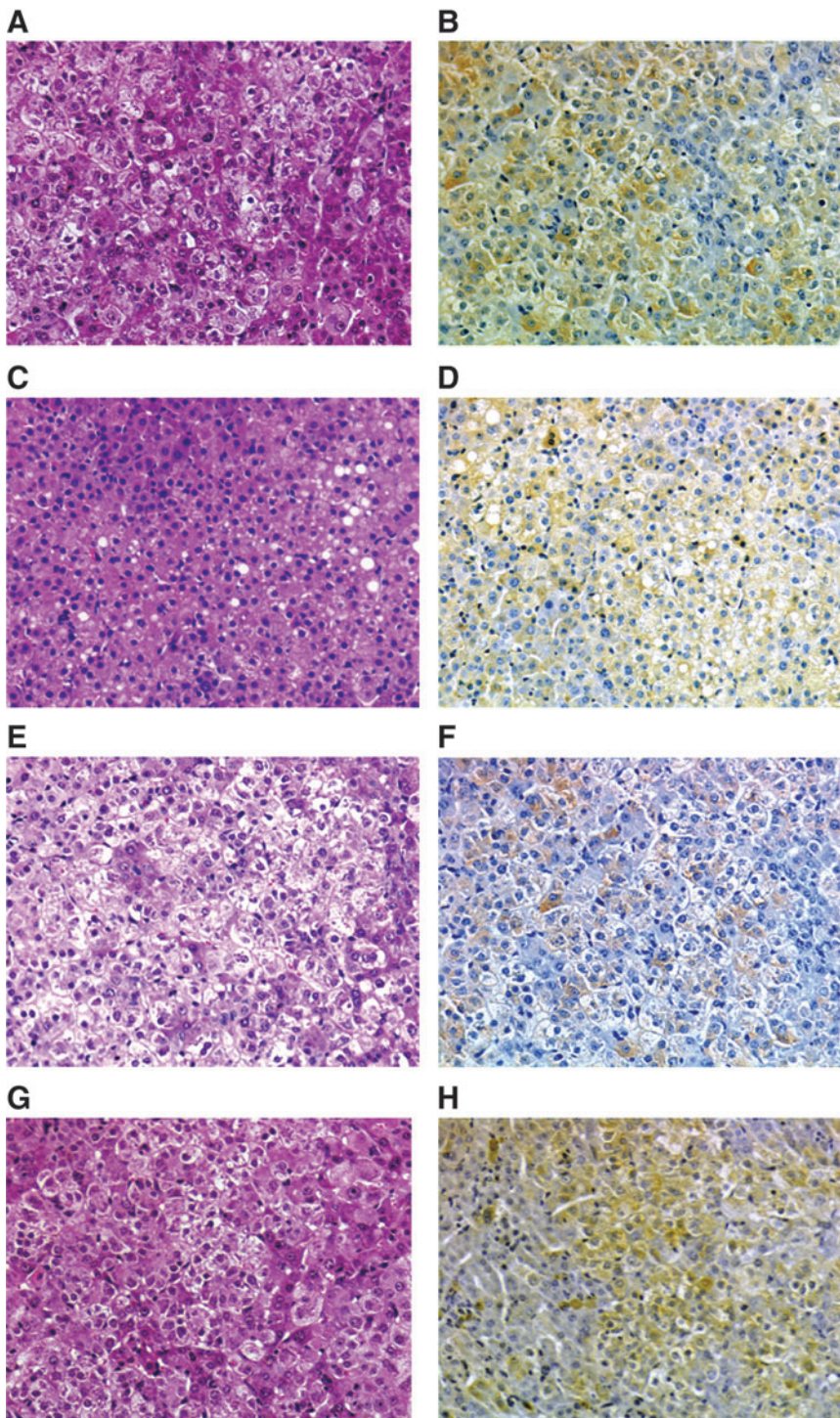


FIG. 6. Histological analysis of the liver of the pCpG-huIFN γ -treated chimeric mice. The livers of HCV-infected chimeric mice that had been untreated (**A** and **B**) or that had been injected with plasmid vectors were collected at 3 days after pCpG-huIFN γ administration (**C** and **D**). In a separate set of experiments, the livers of HCV-infected chimeric mice were collected at 56 days after the administration of pCpG-gLuc (**E** and **F**) or pCpG-huIFN γ (pCpG #3; **G** and **H**). The liver sections were histologically examined either by hematoxylin–eosin staining (**A**, **C**, **E**, and **G**) or immunohistochemical staining with antihuman serum albumin antibody (**B**, **D**, **F**, and **H**). Original magnification, $\times 100$. Color images available online at www.liebertpub.com/humc

are possible. As poorly vascularized regions were observed on occasion in the liver of the chimeric mice, this might be the reason for the poor transfection efficiency and variability in transfection efficiency between mice (Sato *et al.*, 2008). Although the difference in the repopulation rate of human hepatocytes may affect the transgene gene expression level, an apparent correlation between transgene expression level and repopulation rate was not observed. In addition, there was no apparent effect of HCV level in the liver on IFN- γ transgene expression level. A recent study demonstrated that

significant transgene expression is obtained in human liver segments after hydrodynamic injection; however, a very fast injection speed was required compared with that for mice (Herrero *et al.*, 2012). In addition, special attention should be paid when hydrodynamic gene transfer is performed in cirrhotic livers, because the transgene expression level obtained by this method has been shown to be lower in cirrhotic liver in rats (Yeikilis *et al.*, 2006). Under these circumstances, gene transfer to organs other than the liver, such as skeletal muscle, could be an option.

In conclusion, the present study has demonstrated that long-term elimination of human HCV in human hepatocyte chimeric mice with high titers of HCV RNA of genotype 1b can be achieved by IFN- γ exposure after systemic administration of pCpG-huIFN γ . These results indicate that continuous supply of IFN- γ , such as through IFN- γ gene transfer, appears to be a promising antiviral treatment for chronic HCV.

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Author Disclosure Statement

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