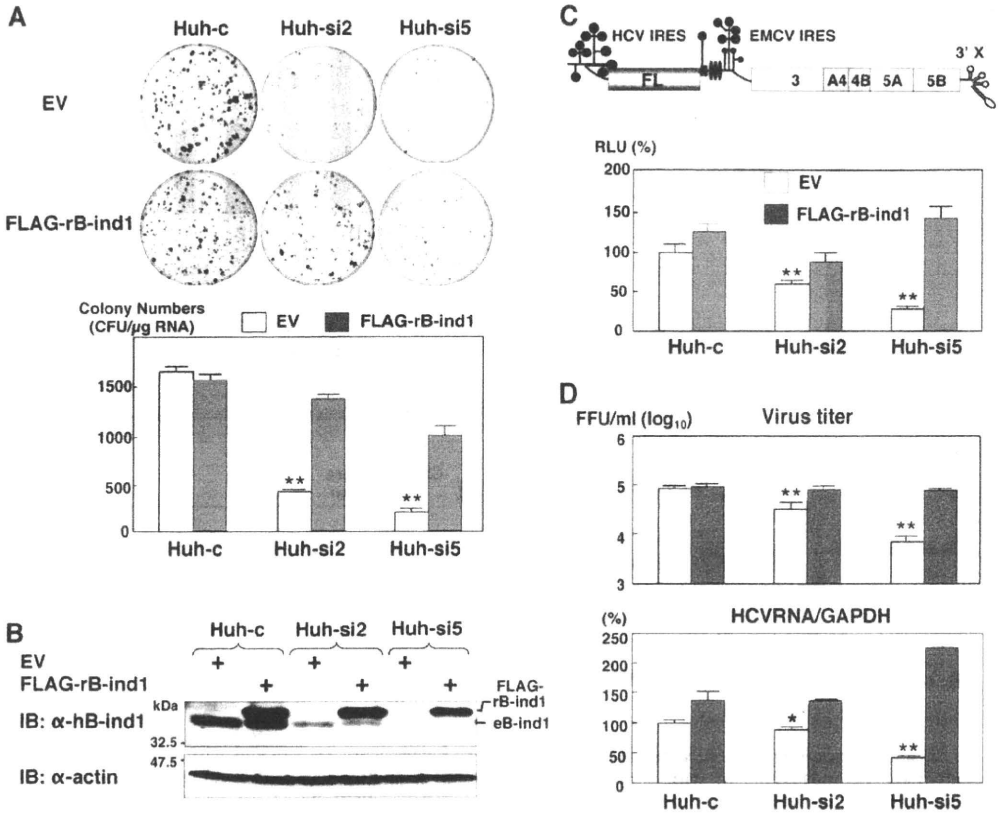

FIG. 4. Effects of hB-ind1 knockdown on the replication of HCV RNA and the production of infectious particles. (A) The hB-ind1 knockdown (Huh-si2 and Huh-si5) and control (Huh-c) cell lines were first transfected with either a plasmid encoding hB-ind1 resistant to siRNA by virtue of the introduction of silent mutations (FLAG-rB-ind1) or an empty vector (EV) and then further transfected with replicon RNA transcribed from pFK-I_{389} neo/NS3-3′/NK5.1. (Upper panel) The cell colonies remaining after cultivation for 4 weeks in the presence of G418 were fixed with 4% paraformaldehyde and stained with crystal violet. (Lower panel) The number of colonies was standardized to the amount of transfected RNA. (B) The expression of the siRNA-resistant hB-ind1 (FLAG-rB-ind1) and the endogenous hB-ind1 (eB-ind1) in Huh-c, Huh-si2, and Huh-si5 cells transfected with either a plasmid encoding FLAG-rB-ind1 or an empty vector was analyzed by Western blotting (IB) with an antibody to hB-ind1 or β-actin. (C) HCV subgenomic replicon RNA transcribed from pFK-I_{389} FL/NS3-3′/NK5.1 and capped *Renilla* luciferase RNA transcribed from pRL-CMV were cotransfected into Huh-c, Huh-si2, and Huh-si5 cells pretransfected with either a plasmid encoding FLAG-rB-ind1 or an empty vector. The firefly luciferase activity was normalized to that of *Renilla* luciferase. HCV IRES-dependent translational activity was expressed as a percentage of the RLU of Huh-c cells transfected with an empty plasmid. EMCV, encephalomyocarditis virus. (D) HCVcc were inoculated into Huh-c, Huh-si2, and Huh-si5 cells pretransfected with either a plasmid encoding FLAG-rB-ind1 or an empty vector. (Upper panel) The culture supernatants at 72 h postinoculation were subjected to a focus-forming assay, and virus titers are expressed as focus-forming units (FFU) per milliliter. (Lower panel) The amount of intracellular HCV RNA was measured by real-time PCR and normalized to the amount of GAPDH mRNA. The HCV RNA level is expressed as a percentage of that of Huh-c cells transfected with an empty plasmid. Data in this figure are representative of three independent experiments. Error bars, standard deviations. Asterisks indicate significant differences (**, $P < 0.01$; *, $P < 0.05$) from the control value.

previously shown that FKBP8 is capable of binding to both NS5A and Hsp90 through the tetratricopeptide repeat (TPR) domain and that the recruitment of Hsp90 to the replication complex plays a crucial role in the replication of HCV (45). Hsp90 is a molecular chaperone and requires various cochaperone proteins such as p23 for efficient chaperone activity. hB-ind1 shows homology to p23 (Fig. 2A), and the FxxW motif, essential for the binding to Hsp90, is conserved in residues $Phe^{107}xxTrp^{110}$ of hB-ind1 (11, 27, 68). To determine whether hB-ind1 interacts with Hsp90 through the FxxW motif as reported for p23, FLAG-tagged hB-ind1 or an hB-ind1 mutant in which Phe^{107} and Trp^{110} had been replaced with Ala (FLAG-hB-ind1AxxA) was coexpressed with HA-tagged Hsp90 in 293T cells and immunoprecipitated with an anti-FLAG antibody. Hsp90 was coimmunoprecipitated with wild-type hB-ind1 but not with the mutant hB-ind1, indicating that hB-ind1 interacts with Hsp90 through the FxxW motif (Fig. 6B).

Previously, we showed that the amino acid residues of the carboxylate clump position in the TPR domain of FKBP8 attach to the C-terminal MEEVD motif of Hsp90 (45). To examine the interaction of hB-ind1 with Hsp90 in the absence of association with FKBP8, FLAG-tagged hB-ind1 was first coexpressed with HA-tagged Hsp90 or mutant Hsp90 lacking the MEEVD motif in 293T cells and then immunoprecipitated with an anti-FLAG antibody. Similar levels of hB-ind1 were coprecipitated with Hsp90 irrespective of the deletion of the MEEVD motif of Hsp90 (Fig. 6C), suggesting that hB-ind1 alone is capable of binding to Hsp90 through the FxxW motif irrespective of the association of FKBP8. To further clarify the interplay among hB-ind1, FKBP8, and Hsp90, FLAG-tagged

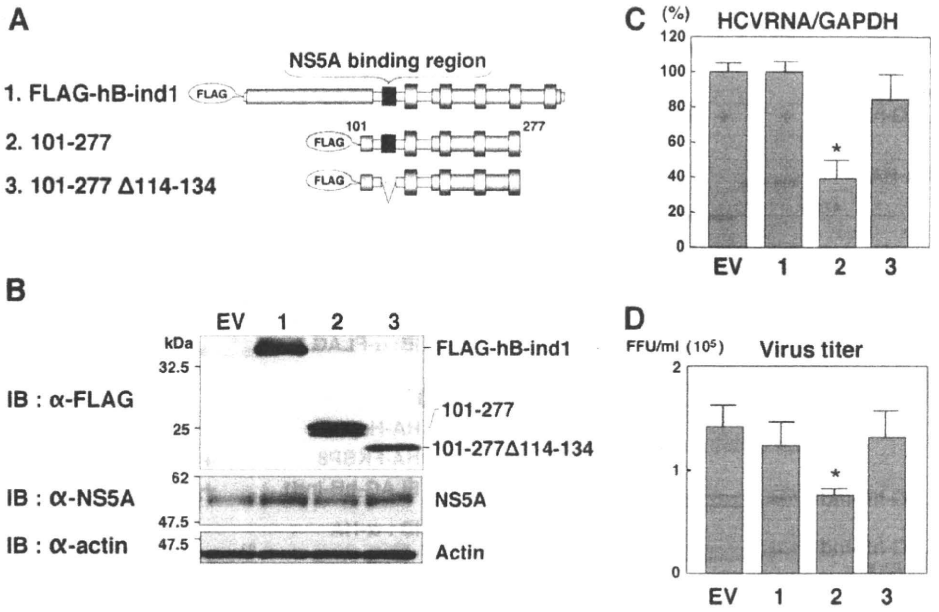

FIG. 5. Dominant-negative effect of an hB-ind1 mutant on the replication of HCV. (A) Plasmids encoding full-length hB-ind1 (construct 1) or deletion mutants of hB-ind1 retaining (construct 2) or lacking (construct 3) the NS5A binding region. (B) One of the three plasmids or an empty vector (EV) was transfected into Huh9-13 cells harboring a subgenomic HCV replicon RNA and was subjected to Western blotting (IB) with specific antibodies at 72 h posttransfection. (C) The amount of intracellular HCV RNA in the Huh9-13 cells was measured at 72 h posttransfection by real-time PCR, normalized to the amount of GAPDH mRNA, and expressed as the percentage of the value for control cells transfected with an empty plasmid. (D) One of the three plasmids or an empty vector was transfected into Huh7.5.1 cells, and then HCVcc were inoculated. Virus production in the culture supernatants at 72 h postinoculation was determined by a focus-forming assay. FFU, focus-forming units. Data in this figure are representative of three independent experiments. Error bars, standard deviations. Asterisks indicate significant differences ($P < 0.01$) from the control value.

hB-ind1 was coexpressed with HA-tagged Hsp90 and/or FKBP8 and then immunoprecipitated with an anti-FLAG antibody. Coprecipitation of Hsp90 with hB-ind1 was increased by additional expression of FKBP8 (Fig. 6D). These results suggest that hB-ind1 interacts with Hsp90 through the FxxW motif and that FKBP8 also participates in the complex formation to enhance the interaction.

hB-ind1 participates in HCV propagation through the interaction with Hsp90. Next, to examine the role of the interaction of hB-ind1 with Hsp90 in the replication of HCV RNA, the replicon RNA transcribed from pFK-I_{389} neo/NS3-3′/NK5.1 was transfected into hB-ind1 knockdown Huh-si5 cells expressing siRNA-resistant FLAG-rB-ind1 or FLAG-rB-ind1AxxA, in which the Hsp90 binding motif FxxW was changed to AxxA. The colony formation in Huh-si5 cells transfected with an empty plasmid was 10% of that in Huh-c cells. The expression of FLAG-rB-ind in Huh-si5 cells recovered the colony formation in Huh-si5 cells to 98% of that in Huh-c cells, although that of FLAG-rB-ind1 AxxA in Huh-si5 cells exhibited only 40% recovery (Fig. 7A). To further examine the role of the interaction between hB-ind1 and Hsp90 in the production of HCVcc, Huh-si5 cells expressing either FLAG-rB-ind1 or FLAG-rB-ind1AxxA were infected with HCVcc, and the virus titer in the culture supernatants and the intracellular HCV RNA level at 72 h postinfection were determined. Virus production was reduced in the culture supernatants, and viral RNA replication in the hB-ind1 knockdown cells was restored by the expression of FLAG-rB-ind1 but not by that of FLAG-rB-ind1AxxA, as seen in colony formation by the replicon RNA (Fig. 7B). Collectively, these results suggest that the interaction of hB-ind1 with Hsp90 through the FxxW motif is required for genomic RNA replication and particle production of HCV.

DISCUSSION

In this study we have shown that hB-ind1 participates in HCV RNA replication and particle production through interaction with NS5A, FKBP8, and Hsp90. hB-ind1 was initially identified as a downstream transducer of Rac1, a member of the small GTP-binding proteins, in mouse fibroblasts treated with sodium butyrate, a multifunctional agent known to inhibit cell proliferation and to induce differentiation by modulating transcription (6, 10). Rac1 possesses diverse biological functions, including cytoskeletal dynamics, membrane ruffling, cell cycle progression, gene transcription, and cell survival (4, 31, 49). Previous studies have suggested that hB-ind1 mediates Rac1 and Jun N-terminal protein kinase–NF-κB signaling and is involved in the regulation of gene expression (6, 10). Inhibition of Rac1 function leads to disruption of cytoskeleton dynamics, resulting in impairment of cell growth (17, 69).

Inhibition of cell growth downregulates HCV RNA replication in the replicon cell line (41, 51), and cell cycle regulation affects HCV IRES-mediated translation (20, 61). Furthermore, cytoskeletal regulation is required for HCV RNA synthesis (3). However, knockdown of hB-ind1 and expression of the deletion mutants exhibited neither morphological change nor suppression of cell growth, suggesting that the suppression

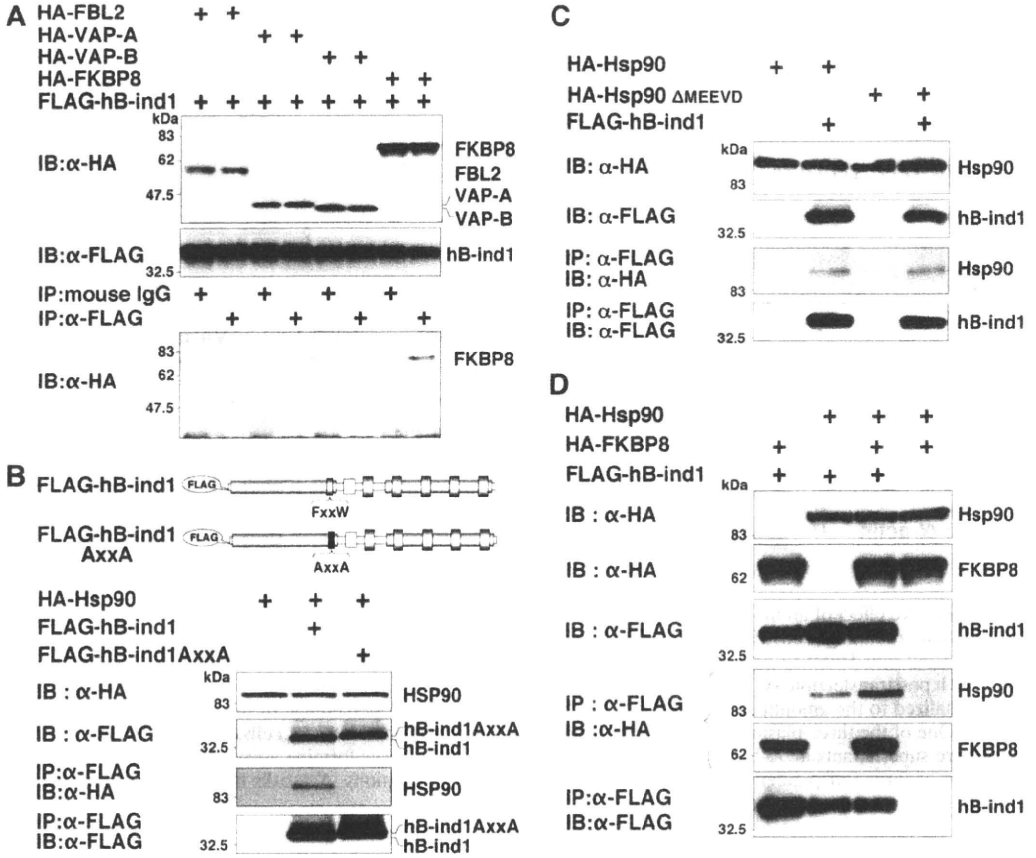

FIG. 6. Interaction of hB-ind1 with other NS5A-binding host proteins. (A) FLAG-hB-ind1 was first coexpressed with HA-tagged FBL2, VAP-A, VAP-B, or FKBP8 in 293T cells and then immunoprecipitated with an anti-FLAG or control antibody. The immunoprecipitates were detected by Western blotting (IB) with an anti-HA antibody. (B) FLAG-hB-ind1 or FLAG-hB-ind1AxxA, in which Phe^{107} and Trp^{110} had been replaced with Ala, was coexpressed with HA-Hsp90 in 293T cells and immunoprecipitated with an anti-FLAG antibody. The immunoprecipitates were detected by Western blotting with an anti-HA or anti-FLAG antibody. (C) FLAG-hB-ind1 was coexpressed with HA-Hsp90 or mutant Hsp90 lacking the MEEVD motif (HA-Hsp90 ΔMEEVD) in 293T cells and was immunoprecipitated with an anti-FLAG antibody. The immunoprecipitates were detected by Western blotting with an anti-HA or anti-FLAG antibody. (D) HA-Hsp90, HA-FKBP8, and FLAG-hB-ind1 were coexpressed in various combinations in 293T cells and immunoprecipitated with an anti-FLAG antibody. The immunoprecipitates were detected by Western blotting with an anti-HA or anti-FLAG antibody. Data in this figure are representative of three independent experiments.

of HCV replication by dysfunction of hB-ind1 is not due to cell growth arrest or cytoskeletal disruption. Murine B-ind1 has been reported to be expressed in all mouse tissues examined, with abundant expression detected in the testis, kidney, brain, and liver (10). Significant levels of endogenous hB-ind1 expression have been detected in the human hepatic cell lines Huh7, HepG2, Hep3B, and FLC4 and in the nonhepatic human cell lines HeLa, 293T, and THP-1 (data not shown); therefore, the tissue specificity of HCV replication could not be explained by the expression of hB-ind1.

Combination therapy with IFN and cyclosporine A has been shown to be effective for patients infected with a high viral load of HCV genotype 1b (24), and cyclosporine A has been shown to suppress HCV RNA replication in vitro through deactivation of the interaction between NS5B and cyclophilin B (66). Cyclophilin and FKBP are classified as immunophilins capable of binding to immunosuppressants cyclosporine A and FK506, respectively (33). The immunophilins do not share a homologous domain with each other, based on their amino acid sequences, substrate specificities, and inhibitor sensitivities. We have recently reported that NS5A binds specifically to FKBP8 but not to other homologous immunophilins such as FKBP52 and cyclophilin D. FKBP8 forms both a homomultimer and a heteromultimer with the chaperone protein Hsp90. Mutation analyses of FKBP8 and Hsp90 suggest that FKBP8 acts as an intermediate between NS5A and Hsp90 via the different position of the TPR domain in FKBP8 and regulates HCV genome replication (45).

The molecular chaperone Hsp90 is one of the most abundant proteins in unstressed cells and generally requires various cochaperone proteins in multiple steps to promote the folding, functional maturation, and stability of its client proteins. Newly synthesized unfolded client proteins are delivered to the Hsp70 complex via Hsp40. In most cases, Hsp70 is able to process the client proteins on its own. Certain substrates require Hsp90 for proper folding or activation. In this case, the scaffold protein Hop connects elements of the Hsp70 and Hsp90 machineries to form an intermediate complex (2, 12, 13, 47). In the late stage, the Hsp70 component dissociates, and at the same time, p23 and immunophilins enter the complex (44, 54) and the

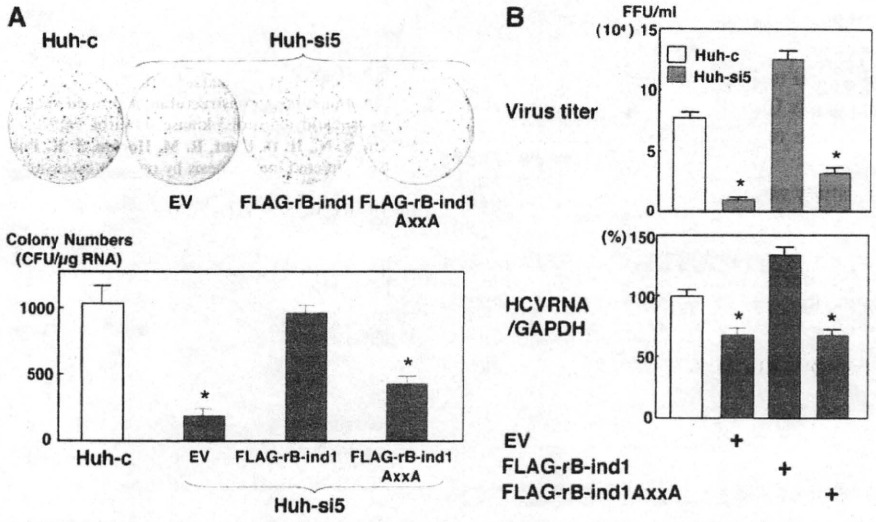

FIG. 7. Role of the interaction of hB-ind1 with Hsp90 in the replication of HCV. (A) hB-ind1 knockdown (Huh-si5) and control (Huh-c) cell lines were transfected either with a plasmid encoding the FLAG-tagged siRNA-resistant hB-ind1 (FLAG-rB-ind1) or FLAG-rB-ind1AxxA (with substitutions in the motif required for binding to Hsp90) or with an empty vector (EV) and were then further transfected with replicon RNA transcribed from pFK-I_{389} neo/NS3-3′/NK5.1. (Upper panel) The cell colonies remaining after cultivation for 4 weeks in the presence of G418 were fixed with 4% paraformaldehyde and stained with crystal violet. (Lower panel) The number of colonies was standardized to the amount of transfected RNA. (B) (Upper panel) Huh-si5 cells expressing either FLAG-rB-ind1 or FLAG-rB-ind1AxxA were infected with HCVcc, and virus production in the culture supernatants at 72 h postinoculation was determined by a focus-forming assay. (Lower panel) The amount of intracellular HCV RNA was measured at 72 h posttransfection by real-time PCR, normalized to the amount of GAPDH mRNA, and expressed as a percentage of the value for control cells transfected with an empty plasmid. Data in this figure are representative of three independent experiments. Error bars, standard deviations. Asterisks indicate significant differences ($P < 0.01$) from the control value.

client proteins are refolded by Hsp90 chaperone activity to achieve the mature form. After that, p23 enhances the dissociation of the mature client protein from the final complex, and the released Hsp90 enters in the next chaperone cycle (72). It has been reported that Hsp90 cochaperone frequencies differ among client proteins (50). FKBP8 interacts with the C-terminal MEEVD motif of Hsp90 through the carboxylate clump position in the TPR domain of FKBP8 (45).

The C-terminal region of hB-ind1 shares homology with PTPLA (60). Protein tyrosine phosphatases are generally involved in the signaling pathways regulating metabolism, cell growth, differentiation, and cytoskeletal dynamics through the conserved $HC(x)_5R$ motif (57). NS5A also interacts with signal transducer and activator of transcription 1 (STAT1) and impairs IFN signaling through the suppression of STAT1 phosphorylation (30). In addition, intracellular uptake of apoptotic cells expressing NS5A by dendritic cells leads to an increase in the secretion of CXCL-8 and impairment of IFN-induced tyrosine phosphorylation of STAT1 and STAT2 (67). Although hB-ind1 lacks the conserved active motif, the interaction of NS5A with the coiled-coil domain in the central region of hB-ind1 may have an effect on the phosphorylation of host proteins involved in the replication of HCV.

Hsp90 has been shown to be involved in the enzymatic activity and intracellular localization of several viral polymerases, including those of influenza virus (39, 42), herpes simplex virus type 1 (5), and Flock house virus (25). Knockdown and treatment with an Hsp90 inhibitor have revealed that Hsp90 activity is important for the rapid growth of negative-strand RNA viruses (9). Furthermore, Hsp90 has been shown to be required for the activity of hepatitis B virus reverse transcriptase (21, 22). Although the precise mechanisms by which Hsp90 and FKBP8 cooperate with NS5A to improve the in vivo replication of HCV have not been clarified yet, treatment with Hsp90 inhibitors in combination with IFN reduced HCV replication in mice xenotransplanted with human liver fragments (43).

In this study, hB-ind1 was shown to interact with Hsp90 through the FxxW motif in the N-terminal p23 homology domain, and the interaction of hB-ind1 with Hsp90 was shown to be further intensified by the expression of FKBP8, suggesting that FKBP8 and hB-ind1 cooperatively recruit Hsp90 to the HCV replication complex. Furthermore, hB-ind1 was shown to be involved in HCV genomic RNA replication and particle production through the interaction with NS5A and Hsp90. These results suggest that hB-ind1 may be involved in the Hsp90 chaperone pathway in a function similar to that of p23 in cooperation with immunophilins such as FKBP8 and that it plays a crucial role in HCV replication in terms of the correct folding of the replication complex required for efficient enzymatic activity. In addition, cyclophilin B may also participate in the translocation of NS5B, as seen in the polymerase subunits of influenza virus, to facilitate binding to the viral RNA. In contrast to cyclosporine A, FK506 per se exhibits no inhibition of RNA replication in HCV replicon cells (65). FKBP8 is a member of the FKBP family but lacks several amino acid residues required for peptidyl-prolyl *cis-trans* isomerase and FK506 binding activities (29). Therefore, nonimmunosuppressive FK506 derivatives that are capable of binding to FKBP8 may exhibit anti-HCV activity. Recently, geldanamycin, an inhibitor of Hsp90, was shown to drastically impair the replication of poliovirus without any escape mutant emerging (15).

Therefore, elucidation of host proteins, including immunophilins, cochaperones, and chaperones, participating in the HCV replication complex may lead to the development of new therapeutics for chronic hepatitis C with a broad spectrum and a low possibility of emergence of breakthrough viruses against antiviral drugs.

In conclusion, in this study we demonstrated that hB-ind1 is involved in HCV replication through interactions with NS5A, FKBP8, and Hsp90. Further clarification of the relationship between viral and host proteins is needed in order to understand the precise mechanism of HCV replication.

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Establishment of an infectious genotype 1b hepatitis C virus clone in human hepatocyte chimeric mice

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The establishment of clonal infection of hepatitis C virus (HCV) in a small-animal model is important for the analysis of HCV virology. A previous study developed models of molecularly cloned genotype 1a and 2a HCV infection using human hepatocyte-transplanted chimeric mice. This study developed a new model of molecularly cloned genotype 1b HCV infection. A full-length genotype 1b HCV genome, HCV-KT9, was cloned from a serum sample from a patient with severe acute hepatitis. The chimeric mice were inoculated intrahepatically with *in vitro*-transcribed HCV-KT9 RNA. Inoculated mice developed viraemia at 2 weeks post-infection, and this persisted for more than 6 weeks. Passage experiments indicated that the sera of these mice contained infectious HCV. Interestingly, a similar clone, HCV-KT1, in which the poly(U/UC) tract was 29 nt shorter than in HCV-KT9, showed poorer *in vivo* infectivity and replication ability. An *in vitro* study showed that no virus was produced in the culture medium from HCV-KT9-transfected cells. In conclusion, this study developed a genetically engineered genotype 1b HCV-infected mouse. This mouse model will be useful for the study of HCV virology, particularly the mechanism underlying the variable resistance of HCV genotypes to interferon therapy.

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INTRODUCTION

Hepatitis C virus (HCV), a positive-sense, single-stranded RNA virus, infects and replicates efficiently only in the hepatocytes of humans and chimpanzees. There are many genotypes of HCV distributed worldwide (Simmonds *et al.*, 1993); among them genotype 1b is the major genotype in Asia, including Japan, and is known to be one of the most resistant genotypes to interferon (IFN) therapy (Fried *et al.*, 2002). Until recently, studies of HCV replication have long been hampered by the lack of a virus culture system. The development of HCV replicon systems has allowed the

The GenBank/EMBL/DDBJ accession numbers for the sequences of HCV-KT9 and HCV-KT1 determined in this work are AB435162 and AB426117, respectively.

study of the mechanisms of replication of HCV (Lohmann *et al.*, 1999). However, these replicons lack structural proteins, do not replicate efficiently without adaptive mutations and do not produce infectious virions. Recently, it was reported that the genotype 2a full-length JFH-1 genome replicated efficiently in Huh7 cells without adaptive mutations and produced virions that were infectious for both naïve cells and chimpanzees, as well as for a human hepatocyte-transplanted chimeric mouse (Wakita *et al.*, 2005; Zhong *et al.*, 2005; Lindenbach *et al.*, 2006). To date, five full-length genotype 1b clones, HCV-N (Beard *et al.*, 1999), Con-1 (Bukh *et al.*, 2002), HCV-J4 (Okamoto *et al.*, 1992), HCV-CG1b (Thomson *et al.*, 2001) and HCV-BK (Takamizawa *et al.*, 1991), have been demonstrated to be infectious by intrahepatic inoculation of transcribed HCV RNA into the liver of chimpanzees. Among these, only the HCV-CG1b genome is reported to produce HCV particles when transfected into Huh7 cells (Heller *et al.*, 2005).

Although the chimpanzee is a useful animal model for the study of HCV infection, there are ethical restrictions on the use of this animal. Instead, Mercer *et al.* (2001) developed a useful small-animal model for the study of HCV infection using chimeric urokinase-type plasminogen activator (uPA)/severe combined immunodeficiency (SCID) mice (which are immunodeficient and undergo liver failure) with engrafted human hepatocytes. This HCV-infected mouse model is reported to be useful for evaluating anti-HCV drugs such as IFN-α and anti-NS3 protease (Kneteman *et al.*, 2006). We have previously described methods to improve the replacement levels of human hepatocytes in this mouse model (Tateno *et al.*, 2004) and we have developed a reverse genetics system for hepatitis B virus (Tsuge *et al.*, 2005) and HCV (Hiraga *et al.*, 2007). In the present study, we report the establishment of an infectious genotype 1b HCV clone that infects and replicates efficiently in human hepatocyte chimeric mice.

METHODS

Cloning of infectious genotype 1b HCV isolate. Serum samples were obtained from a 43-year-old physician who developed severe acute hepatitis after needle stick exposure from a patient with chronic hepatitis C. On admission, the serum total bilirubin concentration was 10.0 mg dl^{-1} and the prothrombin time was 40 %. The patient tested positive for HCV antibodies by a third-generation radioimmunoassay (Ortho-Clinical Diagnostics) and for HCV RNA by RT-PCR. Serum HCV RNA was quantified using an Amplicor Monitor HCV test (Roche Diagnostics). The HCV RNA titre was 2.5×10^6 copies ml^{-1} on admission and then decreased gradually. Fig. 1 shows the serial changes in alanine aminotransferase (ALT) as a measure of liver function and HCV RNA levels in this patient. Serum samples obtained in the early phase of infection were used for cloning the full-length genome.

RNA extraction, cDNA synthesis, plasmid construction and RNA transcription. Total RNA was extracted from 100 µl serum samples using SepaGene RV-R (Sanko Junyaku) and reverse transcribed with random hexamers and ReverTra Ace reverse transcriptase (Toyobo) according to the manufacturer's instructions. PCR primers were designed based on the sequence of HCV-Con1 (GenBank accession no. AJ238799; Bukh *et al.*, 2002). Five overlapping cDNA segments (nt 1–2292, 2269–6715, 6696–9094, 7564–9404 and 9361–9605; nucleotide numbers are those of HCV-Con1) were amplified by PCR with TaKaRa LA *Taq* polymerase (Takara Biochemicals) using the above cDNA. Amplified products were separated by agarose gel electrophoresis. Nucleotide sequences were determined using a Big Dye Terminator Mix Cycle Sequencing kit (Applied Biosystems Japan) with an automated DNA sequencer (model 310; PE Biosystems). We corrected the nucleotide sequences of the obtained clones by site-directed mutagenesis and made them identical to the nucleotide sequences obtained by direct sequencing. Naturally occurring restriction enzyme cutting sites were utilized to clone each segment. We utilized the vector pBR322 and created a multiple-cloning site under the control of the T7 promoter by ligating a linker at restriction enzyme cutting sites as they appeared in order from 5′ to 3′ in the HCV sequences (Fig. 2a). Each segment of HCV was cloned into this vector to generate the full-length clones. The HCV-KT9 clone was established using the 3′-terminal fragment with the longest poly(U/UC) tract length (115 nt), which should have a high replication ability (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003; You & Rice, 2008). A clone with a shorter poly(U/UC) tract length (86 nt), HCV-KT1, was also generated. A polymerase-deficient mutant with an amino acid substitution in the GDD motif (GDD→GND; HCV-KT9-GND) was generated using a Quick Change Site-Directed Mutagenesis kit (Stratagene). After digesting the plasmid with *Xba*I (New England BioLabs) at the 3′ end of the HCV cDNA, HCV RNA was transcribed using T7 RNA polymerase (MEGAscript; Ambion) at 37 °C for 3 h in a 100 µl reaction mixture, according to the manufacturer's instructions. The RNA was analysed using denaturing agarose gel electrophoresis and kept at −80 °C until use.

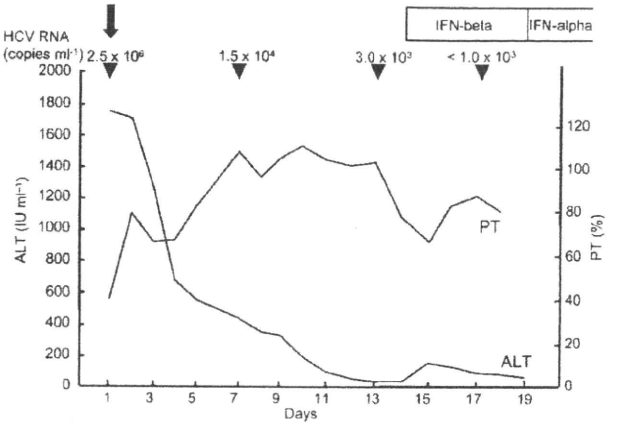

Fig. 1. Clinical course of a patient with severe acute hepatitis C. Alanine aminotransferase (ALT) and prothrombin time (PT) are shown from the day of admission (day 1). The patient was treated daily with 10^6 U IFN-β intravenously for 5 days, followed by 10^6 U IFN-α intramuscularly three times a week for 6 months. HCV RNA was measured on days 1, 7, 13 and 17 (arrowheads). A serum sample was taken on day 1 (arrow) and used to clone the full-length HCV genome.

Construction of a phylogenetic tree. A phylogenetic tree was constructed based on the entire nucleotide sequences of 26 full-length genotype 1b clones plus HCV-KT9. The total number of synonymous and non-synonymous substitutions among the nucleotide sequences was estimated using the method of Gojobori *et al.* (1982) and a phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987).

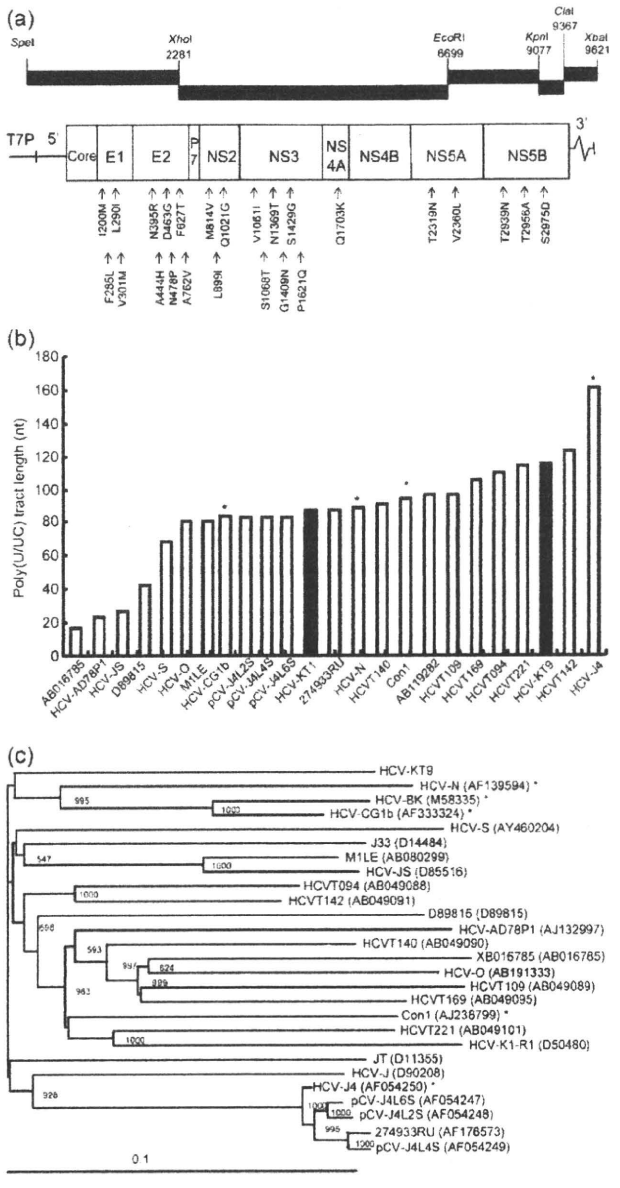

Fig. 2. (a) Schematic diagram of the organization of the cDNA clone HCV-TK9. The T7 RNA promoter (T7P) is located immediately upstream of the HCV genome. Restriction enzyme sites that were used to create clone HCV-KT9 are labelled according to their nucleotide position within the HCV sequence. Amino acid sequences unique to HCV-KT9 compared with 26 other HCV genotype 1b isolates are indicated at the bottom of the figure, with the position of the repaired amino acid residues noted within the polyprotein. (b) Length of the poly(U/UC) tracts of HCV-KT1, HCV-KT9 and 22 other HCV genotype 1b clones reported previously. Asterisks indicate clones confirmed to be infectious by experiments using chimpanzees. (c) Phylogenetic tree constructed with HCV-KT9 and 26 genotype 1b HCV whole-genome sequences. Bar, number of nucleotide substitutions per site. Asterisks indicate clones confirmed to be infectious in experiments using chimpanzees.

Intrahepatic injection experiments in human hepatocyte chimeric mice. We used methods described previously (Tateno *et al.*, 2004) to generate uPA$^{+/+}$/SCID$^{+/+}$ mice and transplant human hepatocytes. All mice used in this study were transplanted with frozen human hepatocytes obtained from the same donor. Mouse serum concentrations of human serum albumin (HSA) correlate with the repopulation index and were measured as described previously (Tateno *et al.*, 2004). Intrahepatic injection of RNA, extraction of serum samples and euthanasia were performed under ether anaesthesia. Briefly, 500 µl RNA solution containing 30 µg transcribed HCV RNA was injected into the liver of anaesthetized chimeric mice through a small abdominal incision. RNA extraction from mouse serum samples, quantification of HCV RNA and nested PCR were performed as described previously (Hiraga *et al.*, 2007). All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments and under the approval of the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University.

Cell culture, RNA transfection and measurement of HCV core antigen. The human hepatoma cell line Huh7 was maintained in Dulbecco's modified Eagle's medium (Sigma) containing 10% fetal calf serum. RNA transfection and measurement of HCV core antigen in the culture medium were performed as described previously (Wakita *et al.*, 2005).

Statistical analysis. The infectious ratio of chimeric mice was compared and the differences assessed using a χ^2 test. Differences in HCV RNA replication ability *in vitro* were analysed statistically by one-way analysis of variance followed by Scheffe's test. A P value of less than 0.05 was considered statistically significant.

RESULTS

Characteristics of genotype 1b clones HCV-KT9 and HCV-KT1

The entire genome of HCV cDNA was assembled from five DNA fragments (Fig. 2a). We obtained 24 3′-extremity clones with different poly(U/UC) tract lengths. We selected the clone with the longest (U/UC) tract because a previous study indicated that the length of poly(U/UC) tract correlates with HCV replication in an HCV replicon system (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003; You & Rice, 2008). The length of the poly(U/UC) tract in the longest 3′ clone was 115 nt. The entire genome length of the HCV-KT9 clone using this longest 3′ clone was 9621 nt. We also generated the clone HCV-KT1 with a shorter (86 nt) poly (U/UC) tract to compare the replication abilities of these clones. The lengths of the poly(U/UC) tracts of 22 clones deposited in GenBank are shown in Fig. 2(b). All infectious clones had a poly(U/UC) tract longer than 80 nt. Fig. 2(c) shows a phylogenetic tree constructed using the nucleotide sequences of the 26 full-length genotype 1b clones published to date. Interestingly, the sequence of HCV-KT9 was closest to that of HCV-CG1b (GenBank accession no. AF333324), which has been reported to be infectious, and formed a cluster with two other infectious clones, HCV-N (Beard *et al.*, 1999) and HCV-BK (Takamizawa *et al.*, 1991). We compared the amino acid

sequences of HCV-KT9 with an alignment of the sequences of the 26 other genotype 1b strains. All HCV full-length clones reported from Japan were included in these 26 strains. Based on these comparisons, we identified 25 aa unique to HCV-KT9 (Fig. 2a). We found that the amino acid sequence of the IFN sensitivity-determining region in the NS5A region, which has been suggested to mediate IFN resistance via interaction with the cellular protein kinase R (Enomoto *et al.*, 1996; Gale *et al.*, 1997), was that of the wild-type.

Intrahepatic injection of HCV-KT1 and HCV-KT9 RNAs into human hepatocyte chimeric mice

In the next experiments, 30 μg *in vitro*-transcribed RNA of HCV-KT1, HCV-KT9 or HCV-KT9-GND was injected into the livers of chimeric mice. Eight of 10 (80 %) HCV-KT9-injected mice developed measurable viraemia at 2 weeks post-inoculation (Table 1 and Fig. 3), with the HCV RNA titre reaching 1.1×10^6 to 8.8×10^6 copies ml^{-1} at 6 weeks post-inoculation (Fig. 3). To check for the presence of infectious HCV in the serum of HCV-KT9-infected mice, each of five naïve mice was injected with 10 μl serum sample (containing 3.5×10^5 copies of HCV) obtained from an HCV-KT9-infected mouse 6 weeks after inoculation. All five naïve mice became positive for HCV RNA, as confirmed by nested PCR, at 2 weeks post-inoculation and two mice developed persistent viraemia (Fig. 4). These results indicated that the serum of HCV-KT9-injected mice contained infectious HCV. In contrast to HCV-KT9, none of the three mice injected with HCV-KT9-GND RNA developed viraemia (Table 1). These results indicated that HCV-KT9 replicates efficiently in mice livers and produces infectious virus continuously. On the other hand, only one out of seven HCV-KT1-injected mice (14 %) developed measurable viraemia (Table 1 and Fig. 3). The level of viraemia was low in this HCV-KT1-infected mouse, HCV RNA was negative by nested PCR at 2 weeks after inoculation and the titre was only 2.2×10^4 copies ml^{-1} at 4 weeks post-inoculation (Fig. 3). These results confirmed the importance of the poly(U/UC) tract length in experimentally induced viraemia.

The nucleotide and amino acid sequences of the viral genome isolated from an HCV-KT9-injected mouse (Fig. 3) at 6 weeks after RNA injection were identical to the injected HCV-KT9 (data not shown). We tried to reclone the poly(U/UC) tract in the HCV-KT1-infected mouse, but it was impossible to reamplify the HCV cDNA using the remaining small amount of serum.

Table 1. Correlation between length of the poly(U/UC) tract and HCV infection

Clone	Length of poly(U/UC) tract	Number of mice			Infection ratio
		Infected	Not infected	Total	
HCV-KT1	86	1	6	7	14 %
HCV-KT9	115	8	2	10	80 %*
HCV-KT9-GND	115	0	3	3	0 %

*P=0.015, compared with HCV-KT1.

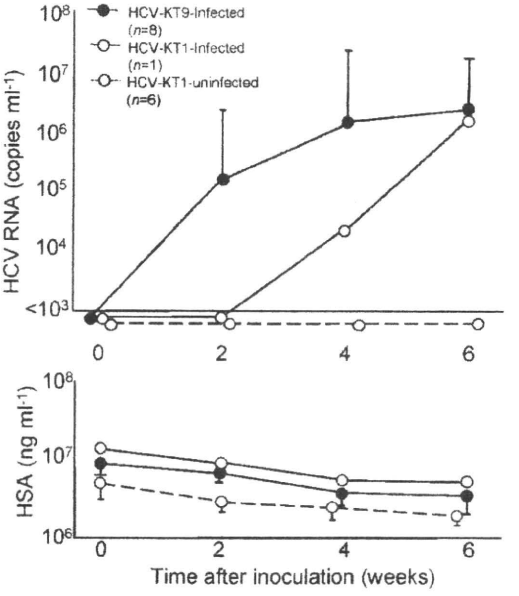

Fig. 3. Changes in HCV RNA levels and HSA concentrations in the sera of mice infected with clonal HCV. Mice were inoculated intrahepatically with 30 μg *in vitro*-transcribed HCV RNA. Eight of the ten HCV-KT9-infected mice (80 %), one of the seven HCV-KT1-infected mice (14 %) and none of the three HCV-KT9-GND-infected mice became positive for HCV RNA. The results for six HCV-KT1-uninfected mice are also shown. Mice serum samples were obtained every 2 weeks post-infection for analysis of HCV RNA titres. Data are shown as mean ± SD.

Analysis of virus production from HCV-KT9-transfected cells

Next, we evaluated the ability of the HCV-KT9 clone to replicate in transfected Huh7 cells. In these experiments, we used JFH-1 RNA, which is known to replicate efficiently in cell cultures, as control (Wakita *et al.*, 2005). Core protein was secreted efficiently from JFH-1 RNA-transfected Huh7 cells. In contrast, we did not observe any measurable levels of core protein in the supernatant of HCV-KT9-transfected cells (Fig. 5), suggesting a minimal replication ability of HCV-KT9 to produce and release virus into the supernatant.

DISCUSSION

In this study, we described the establishment of a genotype 1b clone, HCV-KT9, that replicated efficiently following injection of the transcribed RNA into chimeric mouse liver.

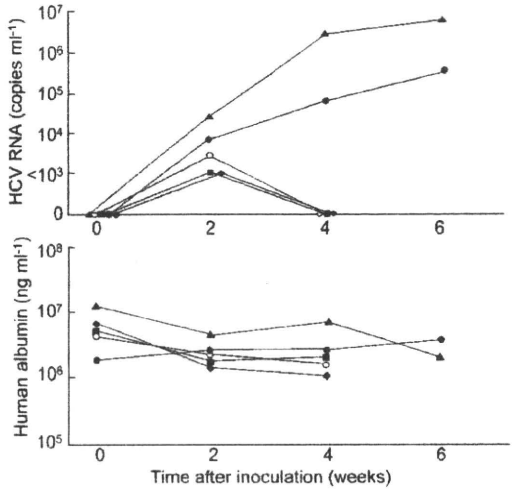

Fig. 4. Passage experiments of HCV in naïve chimeric mice. Five naïve chimeric mice were inoculated intravenously with 10 µl serum sample (containing 3.5×10^5 copies HCV) obtained from an HCV-KT9-infected mouse at week 6 post-inoculation. Serum samples were obtained at the indicated time intervals for the measurement of HCV RNA levels and HSA concentrations. Data represent the changes in five individual mice.

The key factor that determines the infectivity of HCV clones has not yet been established. We previously established a clone from HCV that replicated in a chimeric mouse after injection of serum from a chronically HCV-infected patient. However, we did not observe viraemia after intrahepatic injection of the transcribed RNA from this clone (unpublished results). In contrast, injection of HCV-KT9 RNA in the present study resulted in viraemia in eight out of ten mice (80 %). The fact that the nucleotide and amino acid sequences of the virus recovered from the infected mice were identical to those of the HCV-KT9 clone indicated that no adaptive mutation was necessary for this clone to replicate in the chimeric mouse.

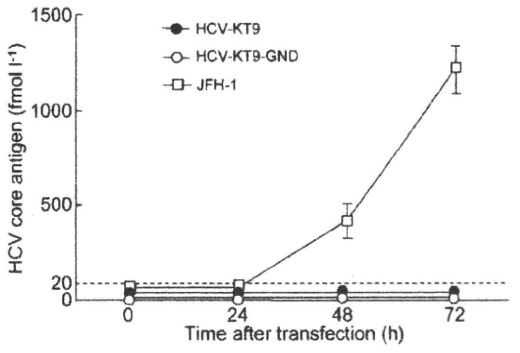

Fig. 5. Time-course studies of HCV core protein secretion into the culture medium of HCV RNA-transfected cells. Huh7 cells were transfected with 10 µg HCV-KT9, HCV-KT9-GND or JFH-1 RNA. HCV core antigen in the culture medium was measured at 24, 48 and 72 h after transfection. Data are shown as mean ± SD of HCV core protein levels obtained from three independent transfection experiments.

Interestingly, the clone was obtained from a patient with severe acute hepatitis. This is similar to JFH-1, an HCV clone with a strong replication ability in cultured cell lines, chimpanzees and chimeric mice, which was cloned from serum samples of a patient who developed acute fulminant hepatitis with a high virus titre (Wakita *et al.*, 2005). A virus that replicates in the early stage of infection may have strong replication ability, which may be lost in the chronic phase of infection.

A key amino acid substitution may be present in one (or some) of the amino acids unique to this clone (Fig. 2a). We also showed that clone HCV-KT1, which differs from HCV-KT9 only in the length of the poly(U/UC) tract, had a poorer replication ability in mice (Table 1 and Fig. 3). However, there is a possibility that a shorter poly(U/UC) tract only slows down the rate of infection, as the HCV RNA titre in the HCV-KT1-infected mouse at 6 weeks after inoculation was similar to that in HCV-KT9-infected mice (Fig. 3). It has been reported that the length and composition of the poly(U/UC) tract is important for the replication of HCV replicons (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003; You & Rice, 2008). However, no replication advantage of a poly(U/UC) tract longer than 86 bp was revealed in this study. This may be due to differences *in vitro* and *in vivo*, where the innate immune response against the virus may be more robust than in cell culture.

As shown in the present study, reverse genetics of HCV has become available for studies of HCV replication. The important factors for virus replication suggested above can be analysed further using this system.

We also examined the response of HCV-KT9-infected mice to IFN treatment. Three HCV-KT9-infected mice were treated with daily intramuscular injections of 1000 IU IFN-α (g body weight)$^{-1}$ for 2 weeks. This regimen resulted in a reduction in HCV RNA levels of only 1.0 log copies ml^{-1} (data not shown). These results are consistent with our previous study, which showed a similar low-level reduction in HCV RNA in mice infected with a genotype 1a clone, and differ from our previous results in mice infected with HCV genotype 2a, which became negative for HCV RNA following daily treatment with 1000 IU IFN-α (g body weight)$^{-1}$ for 2 weeks (Hiraga *et al.*, 2007). These results are in agreement with our clinical experience that genotype 1 is more resistant to IFN therapy than genotype 2. As shown in the present study and previously (Hiraga *et al.*, 2007), reverse genetics of HCV with three genotypes, 1a, 1b and 2a, is now available. By recombination of these clones or the establishment of mutants with nucleotide and amino acid sequences similar to each other, it may be possible to clarify the mechanism underlying the variability in susceptibility of HCV genotypes to IFN.

In this study, HCV-KT9 showed no virus production ability *in vitro*. Recently, Kato *et al.* (2007) reported that the genotype 1b HCV clone CG1b replicated in Huh7.5.1 cells and produced infectious HCV. It will be of interest to create chimeric viruses of HCV-KT9 and HCV-CG1b, and to determine the mutations that are important for virus production *in vitro*.

In summary, we established an infection model of a genotype 1b HCV clone using human hepatocyte chimeric mice. This model will be useful for studies of HCV replication, particularly the mechanism underlying the variable resistance of HCV genotypes to IFN therapy.

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Adoptive immunotherapy with liver allograft–derived lymphocytes induces anti-HCV activity after liver transplantation in humans and humanized mice

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After liver transplantation in HCV-infected patients, the virus load inevitably exceeds pre-transplantation levels. This phenomenon reflects suppression of the host-effector immune responses that control HCV replication by the immunosuppressive drugs used to prevent rejection of the transplanted liver. Here, we describe an adoptive immunotherapy approach, using lymphocytes extracted from liver allograft perfusate (termed herein liver allograft–derived lymphocytes), which includes an abundance of NK/NKT cells that mounted an anti-HCV response in HCV-infected liver transplantation recipients, despite the immunosuppressive environment. This therapy involved intravenously injecting patients 3 days after liver transplantation with liver allograft–derived lymphocytes treated with IL-2 and the CD3-specific mAb OKT3. During the first month after liver transplantation, the HCV RNA titers in the sera of recipients who received immunotherapy were markedly lower than those in the sera of recipients who did not receive immunotherapy. We further explored these observations in human hepatocyte–chimeric mice, in which mouse hepatocytes were replaced by human hepatocytes. These mice unfailingly developed HCV infections after inoculation with HCV-infected human serum. However, injection of human liver–derived lymphocytes treated with IL-2/OKT3 completely prevented HCV infection. Furthermore, an in vitro study using genomic HCV replicon–containing hepatic cells revealed that IFN-γ–secreting cells played a pivotal role in such anti-HCV responses. Thus, our study presents what we believe to be a novel paradigm for the inhibition of HCV replication in HCV-infected liver transplantation recipients.

Introduction

Liver failure and hepatocellular carcinoma (HCC) due to chronic hepatitis C infection are the most common indications for liver transplantation (LT), and the incidences of both have been projected to increase further in the future. Recurrent HCV infection of the allograft is universal, occurs immediately after LT, and is associated with accelerated progression to cirrhosis, graft loss, and death (1, 2). This reflects the suppression of those host-effector immune responses that usually control HCV replication, suggesting that the immunosuppressive environment may play a major role in the rapid progression of recurrent HCV infection after LT (3, 4). Further, the immunosuppressive condition described above is considered to increase the incidence of cancer recurrence after LT in HCC patients. We recently proposed the novel strategy of adjuvant immunotherapy for preventing the recurrence of HCC after LT; this immunotherapy involves intravenously injecting LT recipients with activated liver allograft–derived NK cells (5, 6). Since the immunosuppressive regimen currently used after LT reduces the adaptive immune components but effectively maintains the innate components of cellular immunity (7–9), the augmentation of the NK cell response, which is thought to play a pivotal role in innate immunity, may be a promising immunotherapeutic approach (6). We confirmed that the IL-2/anti-CD3 mAb–treated (IL-2/OKT3–treated) liver allograft–derived NK cells expressed a significantly high level of the tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), which is a critical molecule for tumor cell killing. Further, these cells showed high cytotoxicity against HCC cells, with no such effect on normal cells (5). After obtaining approval from the ethical committee of our institute, we successfully administered adoptive immunotherapy with IL-2/OKT3–treated liver lymphocytes to liver cirrhosis patients with HCC in a phase I trial. Although the long-term benefits of this approach with regard to the control of HCC recurrence after LT remain to be elucidated, this trial provided a unique opportunity to study whether the adoptive administration of IL-2/OKT3–treated liver lymphocytes could also mount an anti-HCV response in HCV-infected LT recipients.

Previous studies have highlighted the important roles of innate lymphocytes in developing immunity against hepatotropic viruses, including HCV (10, 11). In this regard, it is known that patients with chronic HCV infection show diminished NK and NKT cell responses (12–14). In the case of an LT, it has recently been reported that the host CD56$^+$ innate lymphocyte population,

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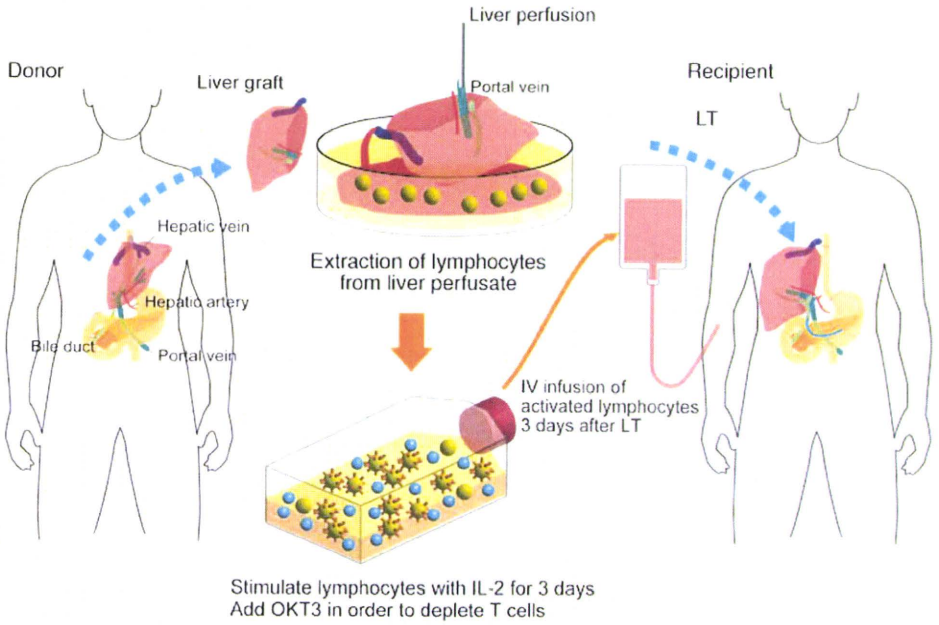

Figure 1
Schematic outline of adoptive immunotherapy with lymphocytes extracted from liver allograft perfusate. The therapy involved giving an intravenous injection of IL-2/OKT3–treated liver lymphocytes to LT recipients. The lymphocytes were extracted from the donor liver graft perfusate. After 3 days of culture with IL-2 (100 JRU/ml), the activated liver NK cell–enriched lymphocytes were administered to the LT recipients through venous circulation. OKT3 (1 μg/ml) was added to the culture medium 1 day before this administration in order to prevent GVHD.

consisting of NK and NKT cells, is appreciably associated with the severity of HCV recurrence after LT (15). These insights into the immunopathogenesis of HCV recurrence indicate that the innate immune components mentioned above are potential targets for therapeutic manipulation. In this study, we have demonstrated for the first time to our knowledge that adoptive immunotherapy with IL-2/OKT3–treated liver lymphocytes, including abundant NK and NKT cells, shows anti-HCV activity after LT, even in an immunosuppressive environment.

Results

Adoptive transfer of IL-2/OKT3–treated liver lymphocytes. The human liver contains a significant number of resident lymphocytes. These cells include abundant $CD56^+$ NK and NKT cells, many of which differ phenotypically and functionally from the circulating cells (14, 16). In our previous study, we performed ex vivo perfusion of the liver through the portal vein, which was necessary in order to flush blood from the liver graft before implantation. Liver-resident lymphocytes were then extracted from the perfusates (number of lymphocytes extracted from normal liver perfusates, 0.5 ± 0.1 cells per gram of liver weight; $n = 14$) (5). Proportions of $CD56^+CD3^-$ NK cells and $CD56^+CD3^+$ NKT cells among the lymphocytes extracted from the liver perfusates (NK cells, 46.4% ± 4.2%; NKT cells, 17.2% ± 2.3%; $n = 14$) were significantly ($P < 0.05$) higher than those among the lymphocytes derived from the peripheral blood of the same donors (NK cells, 21.9% ± 3.7%; NKT cells, 3.8% ± 0.9%; $n = 14$). Extensive preclinical studies have shown that liver allograft–derived resident NK cells mediate remarkably higher cytotoxic activity against HCC cells than do peripheral blood NK cells (5). On this basis, we undertook a clinical trial of adjuvant immunotherapy with IL-2/OKT3–treated liver lymphocytes for preventing the recurrence of HCC after LT in 14 recipients with HCC (Figure 1 and Tables 1 and 2). The therapy involved administering a single intravenous injection of IL-2/OKT3–treated liver lymphocytes to recipients 3 days after LT ($2–5 \times 10^8$ cells injected per subject). In order to prevent graft-versus-host disease (GVHD), i.e., to inactivate $CD3^+$ alloreactive T cells, we added an anti-CD3 mAb, OKT3, to the culture medium a day before the inoculation. During the follow-up period (mean, 23.4 months; range, 10.7–32.9 months), neither any remarkable adverse effects nor rejection episodes occurred. All 14 subjects who received the immunotherapy were alive without recurrence of HCC after LT (including 5 patients with HCC exceeding the Milan criteria; ref. 17). At our institute, the survival rate and recurrence rate of historical control patients with HCC exceeding the Milan criteria were 78% (30 of 37) and 10.8% (4 of 37), respectively. The lymphocytes in the peripheral blood of LT recipients who received immunotherapy in the early postoperative period showed significantly enhanced cytotoxicity against an HCC cell line (HepG2) as compared with those in the peripheral blood of LT recipients who did not receive the therapy in the same period (Figure 2A). Although the gross proportions of NK/NKT cells in the peripheral blood of patients treated with immunotherapy did not differ from those in the peripheral blood of untreated patients, the proportions of $TRAIL^+$ NK cells significantly increased after immunotherapy in the peripheral blood of the former patients. This increase in the $TRAIL^+$ NK cells in the peripheral blood lymphocytes was not observed in untreated patients (Figure 2B). Furthermore, there was a significant correlation between the frequency of $TRAIL^+$ NK cells in the peripheral blood lymphocytes and the NK cytolytic activity of the peripheral blood lymphocytes at 7 days after LT (Spearman rank-order correlation coefficient = 0.54, $P = 0.01$; Figure 2C), indicating the anti-HCC effect of adoptively injected $TRAIL^+$ NK cells. It would be pertinent to conduct additional clinical trials of this immunotherapy for preventing HCC recurrence after LT.

Anti-HCV activity after adoptive immunotherapy. Of the 14 LT recipients who received the immunotherapy, 7 had chronic HCV infection. During the period of this trial, 5 other HCV-infected LT recipients who did not agree to receive immunotherapy served as controls; the background of the controls, including HCV genotype, age, and immunosuppressive therapy, was similar to that of the immunotherapy recipients (Table 3). It has been reported

Table 1
Recipient and tumor characteristics

Patient no.	Age (yr)	Sex	MELD	Hepatitis virus infection	HLA A	HLA B	HLA C	Milan criteria	AFP (ng/ml)	PIVKA-II (AU/ml)	Tumor no.	Maximum tumor size (mm)	Path. vascular invasion	Path. stage	Postop. months	Outcome
1	67	M	19	B	24,-	13,40	03,-	OUT	–	2,584	5	35	–	III	32.9	Alive
2	53	M	16	B	2603,3303	4002,4403	0304,1403	IN	25.3	43	4	11	–	II	31.0	Alive
3	54	M	7	B	0206,3101	3501,5101	0303,1402	OUT	5.7	213	11	26	–	III	29.4	Alive
4	64	F	16	C	2601,2603	3501,4801	0303,-	IN	5.9	142	–	–	–	–	28.5	Alive
5	59	F	14	B	0206,2601	4002,5502	0102,0304	OUT	<5	65	1	13	b1	II	27.8	Alive
6	47	F	8	C	2402,2601	3501,5201	0303,1202	IN	18	46	3	12	–	II	26.2	Alive
7	57	M	29	B	2402,3101	5101,5201	1202,1402	IN	40.3	514	1	25	–	II	25.4	Alive
8	65	F	18	C	1101,2402	5401,5901	0102,-	IN	–	–	3	6	–	II	24.4	Alive
9	60	F	8	–	1101,3001	1302,4006	0602,0801	OUT	32.8	3,026	2	40	vv1	IVA	22.7	Alive
10	56	M	8	C	2402,3303	5201,5801	0302,1202	OUT	–	304	11	22	–	III	19.1	Alive
11	56	M	9	C	0207,-	4601,-	0102,-	IN	47	20	3	25	–	III	17.5	Alive
12	58	M	22	C	1101,3101	1501,3501	0102,0415	IN	–	62	1	17	–	I	16.5	Alive
13	59	M	6	C	1101,2402	1507,1501	0303,0401	IN	202.9	19	3	16	–	II	15.8	Alive
14	51	M	16	B	1101,2601	4002,5401	0102,0304	IN	–	29	–	–	–	–	10.7	Alive

The Milan criteria specifies that liver cancer patients with a single tumor of 5 or fewer centimeters in diameter or 3 or fewer tumors, each no more than 3 cm in diameter, and with no macrovascular invasion, can expect an excellent outcome after LT, with only a 10% risk of cancer recurrence (31). AFP, alpha fetoprotein; F, female; M, male; MELD, model for end-stage liver disease; PIVKA-II, protein induced by vitamin K absence; Path., pathological; Postop., postoperative.

that HCV RNA concentrations sharply decrease a day after LT and increase rapidly thereafter (3). In some of the patients, who did not receive the immunotherapy, HCV RNA titers remained lower than that of the pretransplant titer 1 week after LT, suggesting the individual variation of increasing tempo. However, in almost all patients, HCV RNA titers exceeded the pretransplantation levels by 2 weeks after LT. Notably, HCV infection disappeared in 2 LT recipients after the immunotherapy, but this was not observed in the case of any HCV-infected LT recipients who did not receive the therapy. In one of these patients (who had the lowest HCV RNA levels before LT), HCV RNA has not been detected to date (20 months after LT), even with a qualitative assay. In the other patient, HCV RNA became detectable at 2 months after LT. On the other hand, the 2 patients with the highest HCV viral loads did not respond at all to the immunotherapy. Thus, the effects of immunotherapy were dependent on the HCV virus load before LT, probably because of the proportion of effectors and targets. All patients with HCV viremia are currently being treated with pegylated IFN-α2b and ribavirin. Nevertheless, during the first month after LT, the HCV RNA titers in the sera of LT recipients who received the immunotherapy were statistically lower than those in the sera of LT recipients who did not receive the therapy ($P < 0.05$) (Figure 3). Among the LT recipients who received the immunotherapy, at 2 weeks after LT, HCV RNA remained undetectable in 4 patients (responders), whereas it was detectable in the other 3 patients (nonresponders). The serum ALT levels did not differ between the responders and nonresponders (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI38374DS1), suggesting that the immunotherapy did not inhibit HCV RNA by injuring HCV-infected hepatocytes.

In vitro evidence to prove the anti-HCV activity of IL-2/OKT3–treated liver lymphocytes by using HCV replicon–containing hepatic cells. The liver allograft–derived lymphocytes were cultured in complete medium with and without IL-2 for 3 days. This was followed by adding OKT3 to the culture medium 1 day before coculturing the lymphocytes with HCV replicon–containing hepatic cells in a transwell system, at an indicated time. While the freshly isolated liver allograft–derived lymphocytes inhibited HCV replication in the HCV replicon–containing hepatic cells to some extent, the cultivation of these lymphocytes with IL-2/OKT3 markedly promoted anti-HCV activity. Absence of exposure to either IL-2 or OKT3 resulted in reduced anti-HCV activity of the lymphocytes (OKT3 had a more profound influence than IL-2) (Figure 4A). When the lymphocytes were treated with IL-2 alone, the $CD56^+$ fraction, including NK and NKT cells, that had been isolated by magnetic cell sorting inhibited HCV replication more strongly than the $CD56^-$ fraction; further, the $CD3^-CD56^+$ NK cell and $CD3^+CD56^+$ NKT cell subfractions showed equivalent anti-HCV activity (Figure 4, B and C). On the other hand, when the lymphocytes were treated with both IL-2 and OKT3, the $CD56^+$ and $CD56^-$ fractions showed similar levels of anti-HCV activity (Figure 4B). After the treatment with IL-2 and OKT3, IFN-γ was the predominant cytokine in the culture supernatant of the lymphocytes (Figure 5A), and intracellular IFN-γ expression was induced in the $CD3^-CD56^+$ NK, $CD3^+CD56^+$ NKT, and $CD3^+CD56^-$ T cells (Figure 5B). There was no difference between the proportions of $TRAIL^+$ and $TRAIL^-$ $CD3^-CD56^+$ NK cells producing IFN-γ (Supplemental Figure 2). Adding mAb against IFN-γ to the coculture of lymphocytes with HCV replicon cells markedly weakened the anti-HCV effects. The incomplete restoration of the anti-HCV effect by anti–IFN-γ treat-

Table 2
Donor and graft characteristics

Donor no.	Donor age (yr)	Donor sex	HLA A	HLA B	HLA C	Relationship	Graft	Graft weight (g)	No. of cells administered ($\times 10^6$)
1	41	M	24,-	07,40	03,07	Offspring	Right	608	172
2	24	M	2402,2603	4002,2603	0304,5201	Offspring	Right	658	38
3	51	F	0201,2402	0702,3901	0702,-	Spouse	Right	670	129
4	34	M	2601,2603	4001,4801	0303,0401	Offspring	Left	414	143
5	31	M	0206,2402	4002,5401	0102,0304	Offspring	Posterior	702	135
6	53	F	2402,-	5201,5401	0102,1202	Sibling	Right	538	411
7	24	M	2601,3101	4006,5201	0801,1202	Offspring	Right	642	350
8	34	M	1101,-	4001,5401	0102,1502	Offspring	Right	846	229
9	37	M	0201,1101	1501,4006	0702,0801	Offspring	Left	402	811
10	28	M	1101,3303	5502,5801	0102,0302	Offspring	Right	686	517
11	28	M	0207,2402	4601,5201	0102,5201	Offspring	Right	558	414
12	27	M	0201,1101	1501,3501	0303,0415	Offspring	Right	628	509
13	54	F	1101,2402	1501,1507	0303,0401	Sibling	Right	650	460
14	21	F	2601,2603	1501,5401	0102,0303	Offspring	Right	436	382

ment suggests the possibility that other inflammatory cytokines may also be responsible for the anti-HCV effect, although we have not defined them at present (Figure 5C). Thus, the vigorous anti-HCV activity of IL-2/OKT3–treated liver lymphocytes was dependent, at least in part, on their IFN-γ–secreting activity.

IFN-γ–secreting activity in LT recipients after adoptive immunotherapy. At 14 days after LT, the number of IFN-γ–secreting cells in the peripheral blood of LT recipients who received adoptive immunotherapy was significantly higher than that in the peripheral blood of LT recipients who did not receive immunotherapy during the trial period (Figure 6). This result was consistent with the results of the in vitro studies showing the crucial role of IFN-γ produced in IL-2/OKT3–treated liver lymphocytes.

In vivo evidence to prove the anti-HCV activity of adoptive immunotherapy by using HCV-infected human hepatocyte–chimeric mice. HCV-infected mice have previously been developed by inoculating HCV-infected human serum into chimeric urokinase-type plasminogen activator–SCID (uPA-SCID) mice with engrafted human hepatocytes (18). This HCV-infected mouse model has been reported to be useful for evaluating anti-HCV drugs such as IFN-α and anti-NS3 protease (19). We also generated a human hepatocyte–chimeric mouse model, in which mouse hepatocytes were almost completely replaced by human hepatocytes (20). These mice consistently developed long-term HCV infections, showing high viral titers after inoculation with HCV genotype 1b–infected human serum (50 μl/mouse) (Supplemental Figure 3). Intraperitoneal injection of IL-2/OKT3–treated liver lymphocytes (20×10^6 cells/mouse), at 2 weeks after inoculation with the infected serum, consistently prevented the development of HCV infection in the human hepatocyte–chimeric mice (Figure 7A). Such anti-HCV effects were countered by anti–IFN-γ neutralizing antibodies in some chimeric mice, suggesting the potential role played by IFN-γ in the anti-HCV effects of the immunotherapy. The administration of recombinant human IFN-γ markedly and consistently prevented the development of HCV infection in the human hepatocyte–chimeric mice. Once the HCV RNA became undetectable in the sera of chimeric mice receiving either IL-2/OKT3–treated liver lymphocytes or recombinant IFN-γ, it could not be detected again. The constant levels of human serum albumin in the chimeric mice indicated that neither the immunotherapy nor recombinant IFN-γ administration had significant adverse effects on human hepatocytes in those mice (Figure 7B). Once HCV infection had developed in the human hepatocyte–chimeric mice, who showed high titers of HCV RNA in their sera (over 10^3 copies/ml) 4 weeks after the inoculation of HCV-infected serum, the preventive effects of the adoptive immunotherapy or recombinant IFN-γ on HCV infection were no longer observed (Figure 7C).

Table 3
Characteristics of HCV-infected LT recipients that received and did not receive immunotherapy

No.	Age	Sex	HCV genotype	MELD	Pre–HCV RNA (KIU/ml)	Postoperative months	Immunosuppressant
With immunotherapy							
4	64	F	1b	16	210	29	Basiliximab+FK506+MMF
6	47	F	1b	8	5,000	26	Basiliximab+CsA+MMF
8	65	F	1b	18	2,400	24	Basiliximab+CsA+MMF
10	56	M	1b	8	970	19	Basiliximab+FK506+MMF
11	56	M	1b	9	1,700	17	Basiliximab+FK506+MMF
12	58	M	1b	22	19	17	Basiliximab+FK506+MMF
13	59	M	1b	6	2,200	16	Basiliximab+FK506+MMF
Without immunotherapy							
A	51	M	1b	27	420	42	Basiliximab+FK506+MMF
B	44	M	1b	10	1,600	32	Basiliximab+FK506+MMF
C	54	M	1b	8	180	22	Basiliximab+CsA+MMF
D	56	M	2a	10	470	20	Basiliximab+FK506+MMF
E	57	M	1b	12	3,200	6	Basiliximab+FK506+MMF

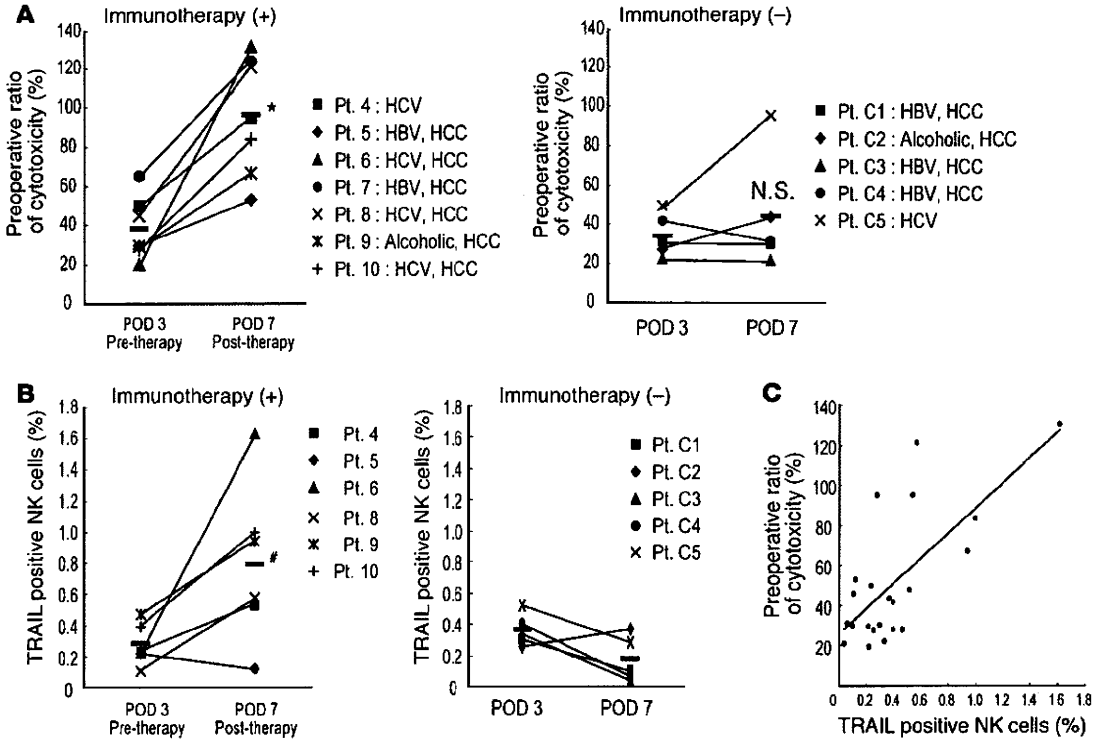

Figure 2

Adoptive immunotherapy with IL-2/OKT3–treated liver lymphocytes promoted the cytotoxic activity and TRAIL expression of NK cells in LT recipients. (**A**) The NK cytotoxic activities of the indicated effectors against their target cells were analyzed by the ^{51}Cr-release assay. The dot plot represents the NK cytotoxic activities of freshly isolated peripheral blood lymphocytes obtained from recipients who received immunotherapy (+) ($n = 7$) and did not receive immunotherapy (–) ($n = 5$) against HepG2 target cells (effector/target [E/T] ratio, 40:1) 3 and 7 days after LT. NK cytotoxic activities are represented as a proportion (percentage) of the preoperative cytotoxicity in each patient. Horizontal lines indicate the mean. Statistical analyses were performed using the 2-tailed, paired Student's t test. *$P < 0.05$ for day 7 versus day 3. (**B**) The frequency of TRAIL$^+$ NK cells increased remarkably in the peripheral blood of LT recipients who received the immunotherapy. Horizontal lines indicate the mean. Statistical analyses were performed using the Mann-Whitney U test. #$P = 0.013$ for immunotherapy group versus untreated group in postoperative day 7. (**C**) Correlation between TRAIL$^+$ NK cell ratio and NK cytolytic activity after LT (Spearman rank-order correlation coefficient = 0.54, $P = 0.01$). Statistical analyses were performed using the Spearman rank-order correlation coefficient. The diagonal line indicates a linear regression line. Each dot indicates the cytotoxicity and TRAIL$^+$ NK cell percentange of each patient. C1, control 1; POD, postoperative day; Pt., patient.

Discussion

The consequences of recurrent hepatitis C on the survival of graft and LT recipients can only be avoided by the development of safe and effective antiviral strategies that can not only prevent initial graft infection but also eradicate established hepatitis C recurrence (3, 4). With regard to initial graft infection, the circulating virions infect the liver graft immediately after LT. HCV RNA concentrations usually increase a few days after LT, reflecting active HCV replication in the liver graft. In general, in such an early phase of a viral infection, the first line of host defense may be effective in removing the virus; however, recent reports have indicated that HCV effectively escapes the innate immune system comprising NK and NKT cells, resulting in persistent infection (21, 22). It has been reported that cross-linking of CD81 on NK cells by the major envelope protein of HCV, HCV-E2, blocks NK cell activation, IFN-γ production, cytotoxic granule release, and proliferation (21). Engagement of CD81 on NK cells blocks tyrosine phosphorylation through a mechanism that is distinct from the negative signaling pathways associated with NK cell inhibitory receptors for major histocompatibility complex class I molecules (22). These facts prove that HCV-E2–mediated inhibition of NK cells is an efficient HCV evasion strategy, which involves targeting the early antiviral activities of NK cells and allowing the virus to establish itself as a chronic infection.

We have explored whether CD81 cross-linking–induced inhibitory effects occur even in IL-2–stimulated NK cells. CD81 cross-linking by a mAb specific for CD81 inhibited antitumor cytotoxicity and anti-HCV activity mediated by resting NK cells, but this manipulation did not alter both these activities of IL-2–stimulated NK cells (Supplemental Figure 4). This indicated that exposure to IL-2 before CD81 cross-linking abrogates subsequent inhibitory signals in the NK cells. This would be one mechanism whereby the adoptive immunotherapy with IL-2/OKT3–treated liver lymphocytes inhibited HCV replication at the early phase of infection after LT.

Although the role of NK cells in controlling HCV infection and replication has not been completely elucidated, a recent report has indicated that NK cells do not exert a direct cytolytic effect on the HCV replicon–containing hepatic cells but release IFN-γ, suppressing HCV RNA expression (11). The role of IFN-γ in the expression of NK cell–mediated anti-HCV activity has been proved by the observa-

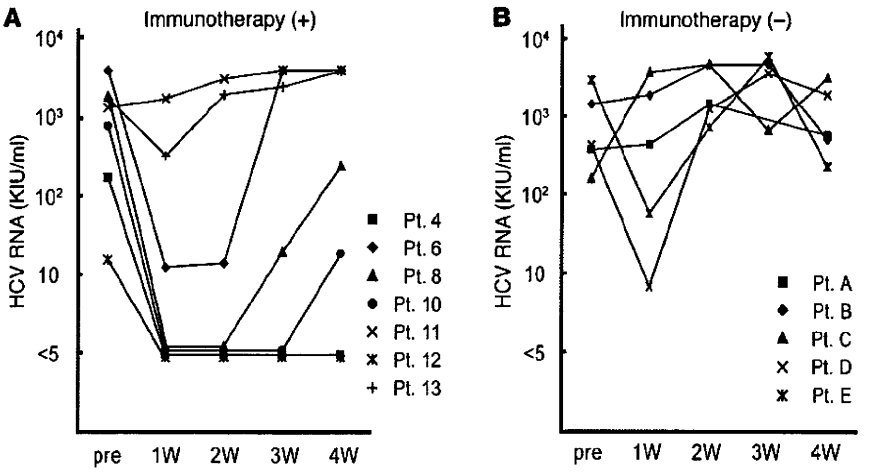

Figure 3
Serial measurement of the HCV RNA titers of LT recipients after LT. The HCV RNA titers in the sera of LT recipients who received immunotherapy were markedly lower than those in the sera of LT recipients who did not receive the therapy during the first month after LT. Each line with a different symbol represents serial HCV RNA titers from an LT recipient who received (+) (**A**; $n = 7$) and 1 who did not receive (–) (**B**; $n = 5$) the immunotherapy after LT. KIU, kilo international unit; pre, pre LT; W, week.

tion that NK cell-conditioned media have an enhanced expression of signal transducer and activator of transcription 1, a nuclear factor that is essential in IFN-γ–mediated antiviral pathways. It has also been reported that hepatocytes cultured in NK cell-conditioned media express higher levels of IFN-α/β, IFN regulatory factor 3, and IFN regulatory factor 7, confirming that NK cells play a key role in suppressing HCV infection of and replication in human hepatocytes in an IFN-dependent manner (23). Similar to recent reports, in the present study, we demonstrated that the NK cells among the IL-2/OKT3–treated liver lymphocytes released soluble factors, predominantly IFN-γ, thus suppressing HCV replication (Figures 5–7).

In addition to NK cells, NKT cells are thought to be involved in eliciting innate responses against infection; however, the role of NKT cells in controlling HCV infection/replication remains unclear. One report has indicated that the number of NKT cells in patients with chronic HCV infection does not differ from that in healthy donors; however, activated NKT cells in HCV-infected patients produce higher levels of IL-13 – but comparable levels of IFN-γ – than those in healthy subjects, showing that NKT cells are biased toward T-helper 2-type responses in chronic HCV infection (24). Another recent report has shown that the sustained response of patients with chronic hepatitis C to treatment with IFN-α and ribavirin is closely associated with increased dynamism of NK and NKT cells in the liver, implicating an NKT cell–mediated mechanism in anti-HCV activity (25). Here, we have described that NKT as well as NK cells in the IL-2/

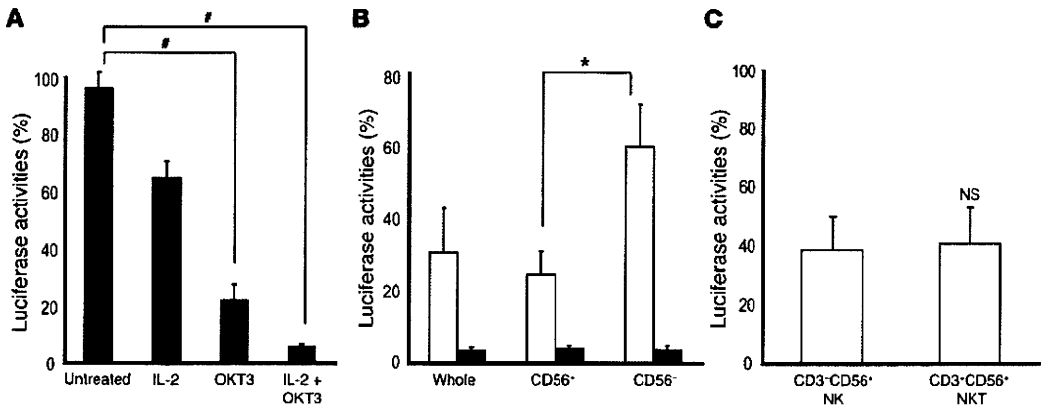

Figure 4
The cultivation of liver lymphocytes with IL-2/OKT3 markedly promoted anti-HCV activity. (**A**) Activation by IL-2 and OKT3 significantly promoted the anti-HCV effect of the liver allograft–derived lymphocytes that were cultured in complete medium with and without IL-2 (100 JRU/ml) for 3 days. OKT3 (1 μg/ml) was then added 1 day before coculturing with HCV replicon cells, at the indicated time. The bar graphs indicate the luciferase activities of the cells in each group. Data are presented as mean ± SEM ($n = 5$). Statistical analyses were performed using the Mann-Whitney *U* test with Bonferroni correction after the Kruskal-Wallis *H* test. #$P < 0.01$ for OKT3 and IL-2/OKT3 treatment versus no treatment. (**B**) $CD56^+$ fraction, including NK and NKT cells, strongly inhibited HCV replication. The culture conditions are described in **A**. By magnetic cell sorting, $CD56^+$ and $CD56^-$ fractions were isolated from the activated lymphocytes and analyzed for anti-HCV activity. The bar graphs indicate the luciferase activities of the cells in each group (IL-2–treated group, white bars; IL-2 plus OKT3–treated group, black bars). Whole, whole lymphocytes. Data are presented as mean ± SEM ($n = 5$). Statistical analyses were performed using the Mann-Whitney *U* test. *$P < 0.05$ for $CD56^+$ fraction versus $CD56^-$ fraction. (**C**) Anti-HCV effect of NK cells was almost identical to that of NKT cells after IL-2 activation. The liver allograft–derived lymphocytes were cultured in complete medium with IL-2 (100 JRU/ml) for 3 days. By magnetic sorting, $CD3^-CD56^+$ (NK) and $CD3^+CD56^+$ (NKT) fractions were isolated from the activated lymphocytes and analyzed for anti-HCV activity. Data are presented as mean ± SEM ($n = 6$).

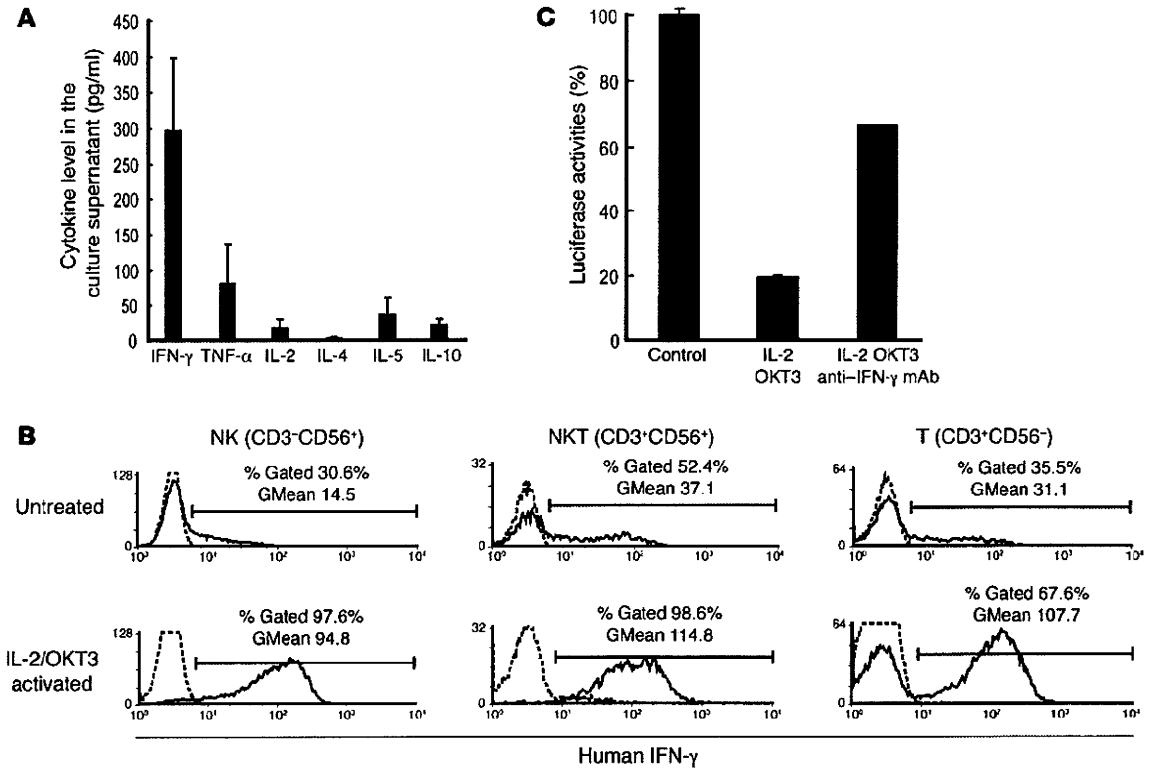

Figure 5

Anti-HCV activity of IL-2/OKT3–treated liver lymphocytes was dependent on their IFN-γ secretion ability. (**A**) IFN-γ was the major cytokine released from the cultured cells. The bar graphs indicate the concentrations of various cytokines (IFN-γ, TNF-α, IL-2, IL-4, IL-5, and IL-10) detected in the coculture supernatant by CBA. Data are presented as mean ± SEM (n = 3). (**B**) The effects of IL-2 and OKT3 (100 JRU/ml and 1 μg/ml, respectively) on IFN-γ production by stimulated $CD3^-CD56^+$ NK, $CD3^+CD56^+$ NKT, and $CD3^+CD56^-$ T cells were evaluated by a combination of cell surface and cytoplasmic mAb staining and subsequent flow cytometric analysis. Histograms represent the log fluorescence intensities obtained upon staining for IFN-γ after gating of each fraction. Dotted lines represent negative control staining with isotype-matched mAbs. Horizontal lines indicate the gated portion of lymphocytes. GMean, geometric mean fluorescent intensity. (**C**) Blocking of IFN-γ with mAb (100 μg/ml) elucidated the marked role played by IFN-γ in producing the anti-HCV effect. The bar graphs indicate the luciferase activities of the cells in each group. Data are presented as mean ± SEM of a representative triplicate sample.

OKT3-treated liver lymphocytes could play a vital role in controlling HCV replication in hepatic cells via an IFN-γ–associated mechanism (Figures 5 and 6).

Therefore, in the early phase of HCV reinfection after LT, the effects of IFN-γ secretion from adoptively injected liver lymphocytes may include inhibition of HCV virion production, which is probably caused by suppression of viral RNA and protein synthesis without immune lysis of intact hepatic cells. This IFN-γ secretion from both $CD3^+CD56^+$ NKT cells and $CD3^+$ T cells was markedly upregulated after treatment with OKT3, which was originally used to prevent GVHD (Figure 5B). This is possibly because of the potent mitogenic activity of OKT3 that induces the activation of $CD3^+CD56^+$ NKT cells and $CD3^+$ T cells. However, the administration of OKT3-coated cells in vivo results in the opsonization and subsequent trapping and/or lympholysis of cells by the reticuloendothelial system (26–28). Thus, GVHD is prevented in LT recipients treated with adoptive immunotherapy.

Our finding that the IL-2/OKT3–treated liver lymphocytes controlled HCV replication via an IFN-γ–associated mechanism can lead to the clinical application of recombinant IFN-γ for anti-HCV treatment. However, a clinically applicable dose of recombinant IFN-γ could not induce significant inhibitory effects on HCV viremia in the previous study (29). Based on the accumulation of adoptively injected IL-2/OKT3–treated liver lymphocytes in the liver of human hepatocyte-chimeric mice (data not shown), the immunotherapy with the liver lymphocytes would provide sufficient IFN-γ to the HCV-infected site.

It has been recently reported that HCV-specific $CD8^+$ T cells exert strong antiviral effects by both cytopathic and IFN-γ–mediated noncytopathic effector functions (30). However, in patients with chronic HCV infection, dysfunction and functional restoration of HCV-specific $CD8^+$ T cell responses have been reported (31). Since HCV-specific $CD8^+$ T cell defects may be important in persistent HCV infections, correcting these defects is considered to our knowledge to be a novel approach to treat HCV infection. Further studies are required to investigate whether activation of NK or NKT cells functionally restores HCV-specific $CD8^+$ T cells.

In conclusion, adoptive immunotherapy using IL-2/OKT3–treated liver lymphocytes containing abundant NK and NKT cells could mount remarkable anti-HCV responses in HCV-infected LT recipients, although its effects were incomplete or transient. Treatment-related improvements, such as defining the best schedule and frequency of cell inoculation and developing more potent effectors, could improve clinical benefits.

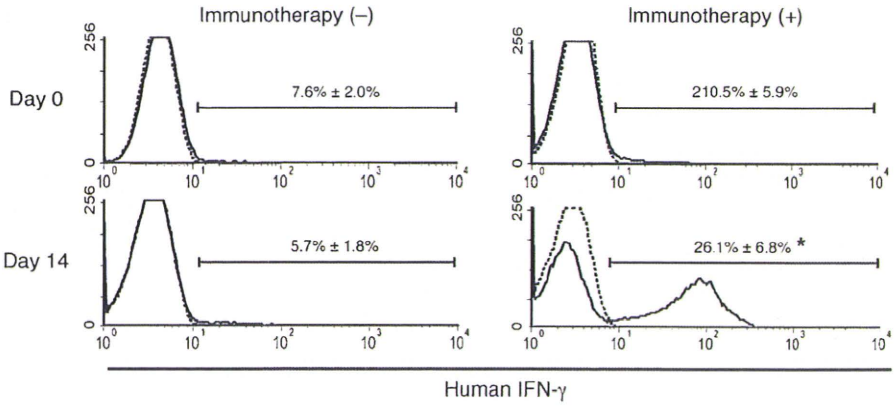

Figure 6

Adoptive immunotherapy with IL-2/OKT3–treated liver lymphocytes induced the production of IFN-γ in the LT recipients. At 14 days after LT, the number of IFN-γ–secreting cells in the peripheral blood of LT recipients treated with the adoptive immunotherapy (+) with IL-2/OKT3–treated liver lymphocytes, including NK and NKT cells, was significantly higher than that in the peripheral blood of untreated LT recipients (–). Histograms represent the proportion (percentage) of IFN-γ–positive cells among the mononuclear cells obtained from the peripheral blood of the immunotherapy (n = 4) and control group (n = 4) LT recipients. Dotted lines represent negative control staining with isotype-matched mAbs. Horizontal lines indicate the gated portion of lymphocytes. Data are presented as mean ± SEM. Histogram profiles shown are representative of 4 independent experiments. Statistical analyses were performed using the Mann-Whitney U test. *P < 0.05 for immunotherapy group versus control group.

Methods

Subjects. All the human liver samples were collected at Hiroshima University Hospital. Tissue specimens were collected after approval from the Institutional Review Board of Hiroshima University and after written informed consent was obtained from the patients. The use of immunotherapy with IL-2/OKT3–treated liver lymphocytes was approved by the Clinical Institutional Ethical Review Board of Hiroshima University. Written informed consent was obtained from all of the patients. This approach was successfully used in 14 cirrhotic patients with HCC undergoing clinical LT (Tables 1 and 2). Of these 14 patients, 7 had chronic HCV infection. Five other LT recipients with chronic HCV infection did not agree to receive this immunotherapy during the trial period. HCV RNA was qualitatively detected in the sera of these patients by a standardized qualitative RT-PCR assay (Amplicor HCV monitor, version 2.0; Roche Diagnostics) every week during the first month after LT.

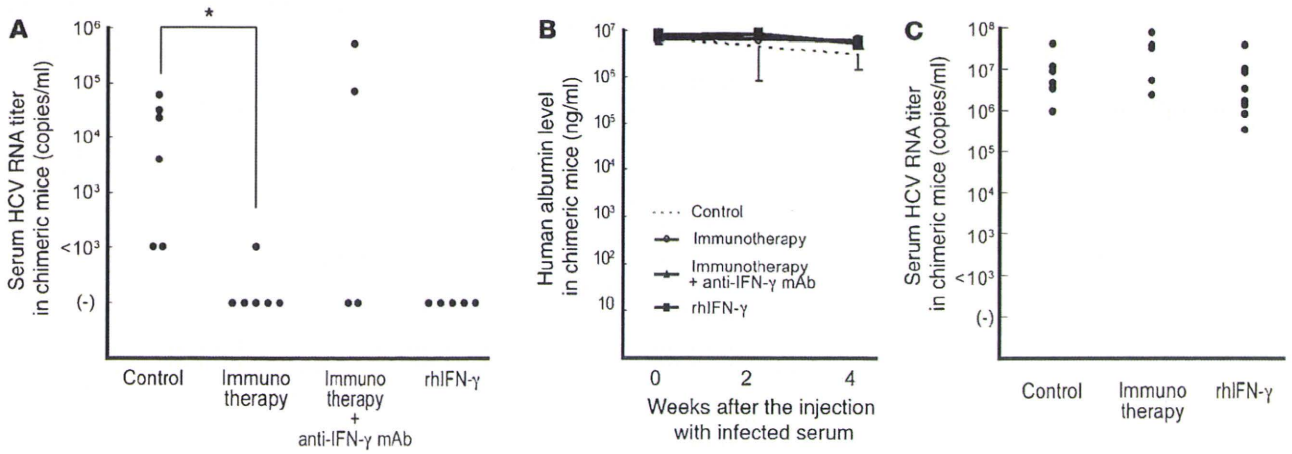

Figure 7

Adoptive immunotherapy with IL-2/OKT3–treated liver lymphocytes prevented HCV infection in human hepatocyte–chimeric mice. (**A**) Human hepatocyte–chimeric mice were intravenously injected with human serum samples positive for HCV genotype 1b. Two weeks after injecting the infected serum, the mice were intraperitoneally inoculated with IL-2/OKT3–treated liver lymphocytes (20 × 10^6 cells/mouse; n = 6) for adoptive immunotherapy. When indicated, anti-human IFN-γ mAb was injected intraperitoneally 1 day before the immunotherapy (n = 4). Intraperitoneal injection of recombinant human IFN-γ (rhIFN-γ) was commenced at 2 weeks after injecting the infected serum (n = 5). The untreated mice served as controls (n = 6). The dot plots represent serum HCV RNA titers in each chimeric mouse 4 weeks after the injecting the infected serum. Statistical analyses were performed using the Mann-Whitney U test. *P < 0.01 for immunotherapy group versus control group. (**B**) The lines represent serial changes in human serum albumin levels in the sera of the mice indicated above. Data are presented as mean ± SEM. (**C**) IL-2/OKT3–treated liver lymphocytes (20 × 10^6 cells/mouse) were intraperitoneally inoculated 4 weeks after the injection with the infected serum (n = 5) for adoptive immunotherapy. Intraperitoneal injection of recombinant human IFN-γ was commenced 4 weeks after the injecting the infected serum (n = 9). The untreated mice served as controls (n = 9). The dot plots represent serum HCV RNA titers in each chimeric mouse 6 weeks after injection with the infected serum.