

**Figure 3.** Relationship between increase in the number of hypermutated genomes and hepatitis B virus (HBV) e antigen (HBeAg) levels in 15 HBeAg-positive patients with chronic HBV infection who experienced  $>2$  increases of  $>100$  IU/L in alanine aminotransferase (ALT) level. Patients' exacerbations were divided into 2 groups, A and B, according to the extent of increase in the number of hypermutated genomes, relative to the basal number (group A included 9 exacerbations that involved a  $>5$ -fold increase in the number of hypermutated genomes; group B included 8 exacerbations that involved a  $\leq 5$ -fold increase in the number of hypermutated genomes). *Upper panel* for groups A and B, individual e antigen levels at the time the ALT level increased and 2 months later; in the upper panel for group A, *dashed lines* indicate 4 exacerbations associated with seroconversion to positivity for antibody against e antigen. *Lower panel* for groups A and B, box-and-whisker plots for e antigen levels at these same 2 time points. In the plots, the lines in the boxes indicate median values; the upper and lower lines of the boxes indicate the 25th and 75th percentiles, respectively; and the upper and lower whiskers represent the 90th and 10th percentiles, respectively.

supernatant (figure 5A and 5B). These results suggest that the infectivity (or replication ability) of HBV with hypermutated genomes is very poor. It is possible that the inoculum contained less abundantly mutated genomes. To test this, we cloned and sequenced 72 clones of 217-bp DNA fragments amplified at a denaturation temperature of  $95^{\circ}\text{C}$ . Of 72 clones obtained from the inoculum, we found 1 clone with 8 G-to-A substitutions, 1 clone with 5 substitutions, 2 clones with 3 substitutions, and 1 clone with 1 substitution (figure 5C). In contrast, 1 of the 72 clones obtained from the mouse serum had 1 G-to-A substitution. If G-to-A substitutions were excluded, the only other nucleotide substitution observed in the 144 clones sequenced was a single C-to-T substitution.

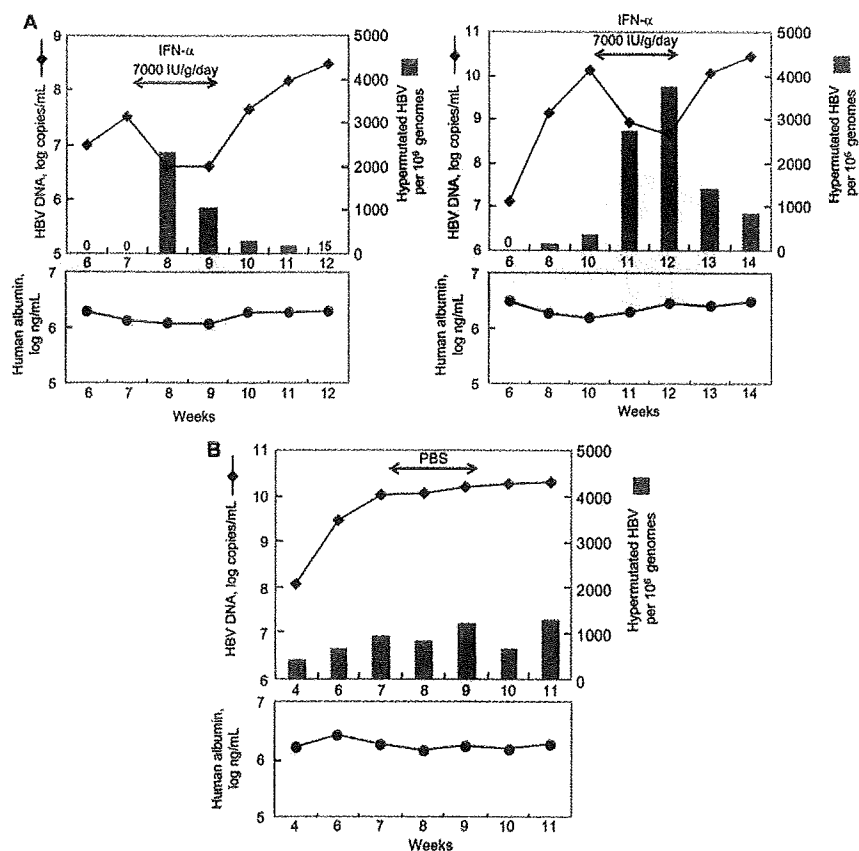
## DISCUSSION

In a previous study, we found that the majority of serum samples obtained from HBV-infected patients contained a small number of hypermutated genomes [27]. Recently, we developed a method (TaqMan 3D-PCR) to measure small numbers of hypermutated genomes [28]. Using this method, we reported dual antiviral effects for APOBEC3G, namely induction of hypermutation and reduction of viral replication. We also reported that

IFN increased the transcription of APOBEC3G and enhanced the effect of the protein *in vitro* [28]. Other investigators also showed that IFN enhances the action of APOBEC proteins against HIV [18–21]. It is thus assumed that the antiviral effect of APOBEC proteins should be enhanced by IFN and other cytokines *in vivo*.

In the present study, we showed that an increase in ALT level accompanied by an increase in the number of hypermutated genomes was associated with reduction in the plasma HBV DNA level. In contrast, no decrease in HBV DNA level was observed if the increase in ALT level occurred in the absence of an increase in the number of hypermutated genomes. It is difficult to know which of the dual antiviral effects of APOBEC3G (or other APOBEC proteins) reduced the viral level. It is also impossible to estimate the importance of APOBEC proteins in this reduction. However, it is clear that the increase in the number of hypermutated genomes of HBV correlates with activation of the host antiviral defense against HBV.

We also demonstrated that exacerbations of HBV infection associated with a marked increase in the number of hypermutated genomes were associated not only with a decrease in the plasma HBV DNA level but also with clearance of e antigen.

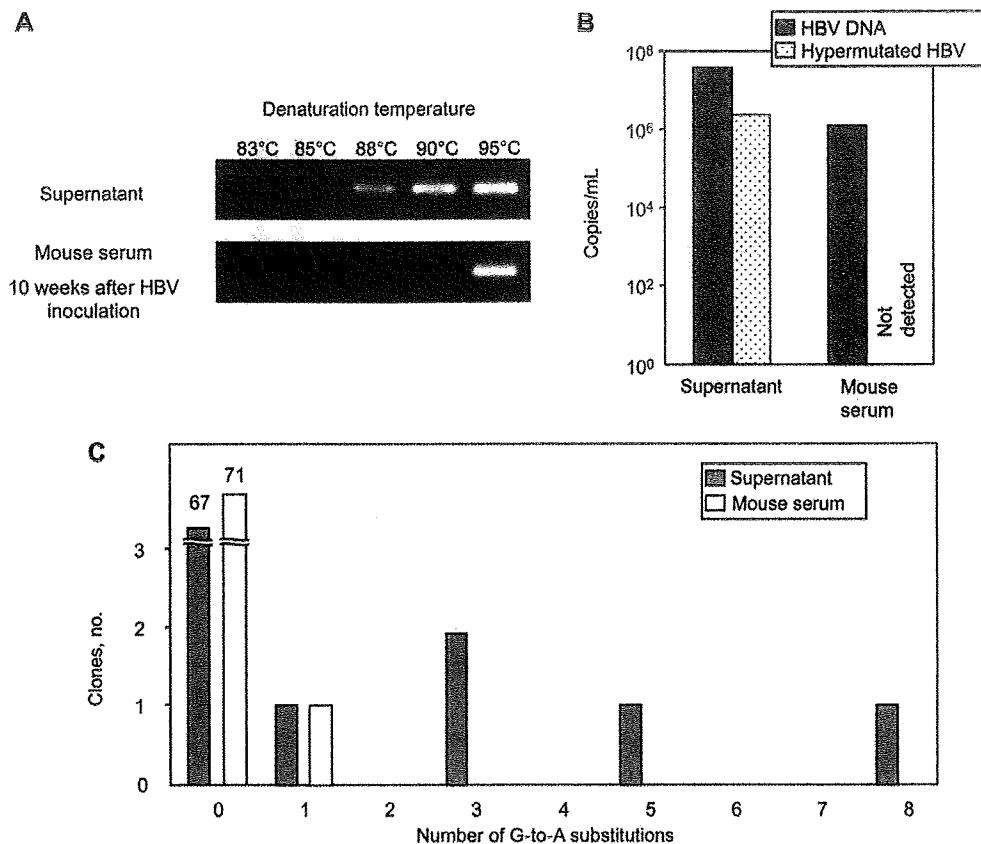


**Figure 4.** Effect of interferon (IFN)- $\alpha$  therapy on hepatitis B virus (HBV) hypermutation in HBV-infected, human hepatocyte-chimeric mice. Two chimeric mice (A) were inoculated with recombinant wild-type HBV produced by transfected HepG2 cells; 10 weeks later, after confirmation of high-level HBV viremia, they were treated with IFN- $\alpha$  at 7000 IU/g/day for 14 days, by intramuscular injection. Upper panels in both parts of A, serum HBV DNA levels and the number of hypermutated genomes; lower panels in both parts of A, human serum albumin concentrations. Note that the albumin levels are stable during IFN- $\alpha$  therapy. A control mouse (B) was inoculated with recombinant wild-type HBV produced by transfected HepG2 cells and treated with phosphate-buffered saline (PBS). Upper and lower panels of B show the same information as in A.

Furthermore, all exacerbations followed by seroconversion to positivity for antibody against e antigen were associated with a marked increase in the number of hypermutated genomes. Clearance of e antigen often results from a G-to-A nucleotide substitution at the first position of a 5'-GGGG stretch in the pre-core coding sequence (the G1896A mutation). Because this substitution (changing TGGGG to TAGGG) is in agreement with the dinucleotide pattern preferentially edited by APOBEC3G, one might assume that G-to-A substitution in this region could be caused by this enzyme and is related to the clearance of e antigen. However, we observed that hypermutation was induced in only some genomes, whereas the majority of genomes were unaffected. Thus, it seems unlikely that APOBEC proteins play a role in seroconversion to positivity for antibody against e antigen, although it is still possible that the 5'-GGGG stretch in the precore region is the preferred editing site for the enzyme. Importantly, such substitution of the 5'-GGGG stretch should result in the occurrence of multiple stop codons (TAG, TGA, and TAA) in HBV genomes, as we observed and reported in our

previous study [28], which makes the replication of mutated genomes impossible.

In the present study, we did not observe any increase in the number of hypermutated genomes during IFN therapy in some patients. This finding is discrepant from the results of previous in vitro experiments that showed increased numbers of hypermutated genomes after the application of IFN [28]. Interestingly, our experimental results also showed the induction of APOBEC3G gene expression, an increase in the number of hypermutated genomes, and a reduction of plasma HBV DNA level in 2 human hepatocyte-chimeric mice treated with IFN (figure 4). What is the reason for the lack of increase in hypermutation in some IFN-treated patients? We usually administer IFN to patients who have high ALT levels. The patients in this study had abnormal ALT levels prior to treatment with IFN—that is, their livers were inflamed, and the levels of many cytokines produced by the immune cells in the liver were already high. We presume that the effect of these elevated cytokine levels masked the effect of the IFN we administered. It could also be argued that the effect



**Figure 5.** Results for a human hepatocyte-chimeric mouse inoculated with hepatitis B virus (HBV) produced by HepG2 cells transfected with an equal amount (5  $\mu$ g each) of HBV and apolipoprotein B messenger RNA editing enzyme, catalytic polypeptide-like 3G plasmids. The inoculum contained ~6.25% hypermutated genomes. A serum sample was obtained 10 weeks after the inoculation. *A*, HBV DNA was amplified by polymerase chain reaction (PCR) that used different denaturation temperatures and run on 2% agarose gel. *B*, Quantitative measurement of HBV DNA and hypermutated DNA in the inoculum and mouse serum. *C*, Number of G-to-A substitutions found in each of 72 clones obtained from products of PCR of culture supernatant or mouse serum.

observed in mice represents the absence of the immune response in mice, whereas the lack of a clear response to IFN in the study patients was the result of the complex immune response in human beings. Alternatively, the concentrations of IFN in treated patients might be lower than those used for the cell culture or the chimeric mice. Although we did not perform this analysis in the present study, it would be interesting to determine the expression levels of APOBEC proteins and IFN-stimulated genes in the liver of IFN-treated patients.

The present study showed that the number of hypermutated genomes increased during some increases in ALT level, probably as a result of IFN-activated APOBEC proteins and other cytokines in patients with chronic hepatitis B. However, the number of hypermutated genomes was very small, only 28,378 in 10<sup>6</sup> HBV genomes at most (figure 1A). Because it was possible that the less abundantly hypermutated genomes were not detected (i.e., that genomes with only 1 or 2 G-to-A substitutions were not amplified by 3D-PCR), cloning and sequencing were performed to detect such genomes. However, the number of ge-

nomes containing G-to-A substitutions was still low (5 [6.9%] of 72 clones), even in the culture medium of HepG2 cells cotransfected with APOBEC3G and HBV (figure 5C). This means that the number of genomes with only a small number of G-to-A substitution was not high, suggesting that only selected DNA molecules were heavily mutated while the remaining DNA was not. Does this mean that the effect of APOBEC proteins in antiviral defense is trivial in patients with chronic HBV infection? It is a possible that the heavily deaminated genomes are an easy target for uracil DNA glycosylase. Although the dual antiviral effects of APOBEC proteins are currently known to reduce the amount of HBV, the importance and magnitude of APOBEC proteins with respect to in vivo virus reduction should be investigated further.

Treatment of patients with chronic HBV infection has improved with the advent of new nucleoside and nucleotide analogues. However, reactivation of HBV and flare-ups of hepatitis are often seen in patients who stop such therapy. Furthermore, hepatitis B surface antigen clearance is rare in patients treated

with these antiviral drugs. On the other hand, most patients with chronic HBV infection achieve sufficient viral suppression and disease quiescence through immunological suppression of the virus. As we showed in this study, the immunological suppression of HBV is much stronger than that achieved with IFN therapy, but it is often transient. It is thus necessary to clarify the mechanism of transient immune response and to develop treatment that produces persistent suppression of HBV. Quantitative measurement of hypermutated genomes should be useful in monitoring the immune response in this context.

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## Amphipathic DNA Polymers Inhibit Hepatitis C Virus Infection by Blocking Viral Entry

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See editorial on page 427.

**BACKGROUND & AIMS:** Hepatitis C virus (HCV) gains entry into susceptible cells by interacting with cell surface receptor(s). Viral entry is an attractive target for antiviral development because of the highly conserved mechanism. **METHODS:** HCV culture systems were used to study the effects of phosphorothioate oligonucleotides (PS-ONs), as amphipathic DNA polymers (APs), on HCV infection. The *in vivo* effects of APs were tested in urokinase plasminogen activator (uPA)/severe combined immunodeficient (SCID) mice engrafted with human hepatocytes. **RESULTS:** We show the sequence-independent inhibitory effects of APs on HCV infection. APs were shown to potently inhibit HCV infection at submicromolar concentrations. APs exhibited a size-dependent antiviral activity and were equally active against HCV pseudoparticles of various genotypes. Control phosphodiester oligonucleotide (PO-ON) polymer without the amphipathic structure was inactive. APs had no effect on viral replication in the HCV replicon system or binding of HCV to cells but inhibited viral internalization, indicating that the target of inhibition is at the postbinding, cell entry step. In uPA/SCID mice engrafted with human hepatocytes, APs efficiently blocked *de novo* HCV infection. **CONCLUSIONS:** Our results demonstrate that APs are a novel class of antiviral compounds that hold promise as a drug to inhibit HCV entry.

Hepatitis C virus (HCV) infects approximately 200 million people worldwide.<sup>1</sup> The majority of HCV-infected patients fails to clear the virus, and many develop chronic liver disease including cirrhosis with a risk of hepatocellular carcinoma. Treatment of chronic hepatitis C is currently based on peginterferon-alfa and ribavirin, which is accompanied by substantial adverse effects and is only effective in approximately half of the patients.<sup>2,3</sup> In addition to other viral targets, viral entry is an attractive target for antiviral development because of the

potentially conserved mechanism of viral entry.<sup>4</sup> Although several candidate receptors for HCV have been identified,<sup>5-10</sup> the mechanism of HCV entry still remains largely unknown. Previous reports have indicated a pH dependency for entry of HCV pseudoparticles (HCVpp) as well as cell culture-generated HCV (HCVcc), suggesting that HCV enters cells by receptor-mediated endocytosis.<sup>7,11,12</sup> Antiviral compounds targeting the entry step of viral infection have been successfully developed in other viral infections.<sup>13</sup> Recent studies have shown that phosphorothioate oligonucleotides (PS-ONs), as amphipathic DNA polymers (APs), have a sequence-independent antiviral activity against human immunodeficiency virus type 1 (HIV-1) as entry inhibitors.<sup>14</sup> The antiviral effect of APs appears to be specific to the phosphorothioate backbone, which confers an amphipathic structure, because the phosphodiester oligonucleotides (PO-ONs) as nonamphipathic polymers are ineffective.<sup>14</sup>

### Materials and Methods

#### Cell Culture and Oligonucleotide Synthesis

Huh7.5 (provided by Charles Rice), Huh7.5.1 (provided by Francis Chisari), Huh7, and Hep3B cells were maintained at 37°C, 5% CO<sub>2</sub> in Dulbecco's modified Eagle medium, containing 10% fetal bovine serum. All PS-ONs and PO-ONs were synthesized as described previously.<sup>14</sup> Oligonucleotides lacking the phosphorothioate modification (PO-ONs) were synthesized with the addition of 2'-O-methyl ribose modification, which stabilizes oligonucleotides from nuclease degradation.<sup>14</sup> Compounds used in the *in vivo* experiment were synthesized under good manufacturing practice (GMP) conditions to yield high-purity sodium salts.

**Abbreviations used in this paper:** APs, amphipathic DNA polymers; HCV, hepatitis C virus; HCVcc, cell culture-generated HCV; HCVpp, HCV pseudoparticles; VSVGpp, vesicular stomatitis virus G protein pseudoparticle; PO-ON, phosphodiester oligonucleotide; PS-ONs, phosphorothioate oligonucleotides.

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### HCV Infection and Replication Assays

The production of cell culture-generated HCV JFH-1 (HCVcc) and HCV pseudovirus (HCVpp) has been reported previously<sup>5,15</sup> and is described in detail in the Supporting Document. HCVpp harboring E1/E2 glycoproteins from genotypes 1a, 1b, 2a, 3a, 4a, 5a, and 6a were described previously.<sup>16</sup> For viral internalization assay, Hep3B cells were incubated for 1 hour at 4°C to allow binding of HCVpp (pHCV7a) to cells, washed repeatedly with phosphate-buffered saline to remove unbound virus, and treated with concanamycin A (Sigma-Aldrich, St. Louis, MO) (25 nmol/L), Anti-E2 AP33 antibody<sup>17</sup> (25 µg/mL), PS-ON (100 nmol/L), or PO-ON (100 nmol/L) overnight at 37°C for viral entry. The efficiency of infection was measured by luciferase assay 24 hours later. Transient assay of genotypes 1b (Con-1) and 2a (JFH-1) subgenomic reporter replicons have been reported previously<sup>18,19</sup> and are described in detail in the Supporting Document.

### HCV Binding and Fusion Assays

The HCV-like particle (LP) binding assay was performed at 4°C for 1 hour in 100 µL of TNC (50 mmol/L Tris, pH 7.4, 100 mmol/L NaCl, 1 mmol/L CaCl<sub>2</sub>) buffer containing 1% bovine serum albumin as reported previously<sup>20</sup> and is described in detail in the Supporting Document. Both Hep3B and Huh7.5 cells were tested. Direct binding of PS-ON or PO-ON to HCV-LP was measured by a plate-binding assay and is described in the Supporting Document. For viral fusion assay, HCVpp/liposome lipid mixing assays with rhodamine-labelled liposomes were performed as previously reported<sup>21</sup> and are described in the Supporting Document.

### HCV Infection in Chimeric Mice

Human hepatocyte-transplanted mice generated in severe combined immunodeficient (SCID)/urokinase plasminogen activator (uPA) mice were purchased from PhenixBio (Hiroshima, Japan).<sup>22</sup> These uPA/SCID mice stably transplanted with human hepatocytes were treated intraperitoneally with 10 mg/kg of poly C PS-ON or poly AC PS-ON (40mer) on days -1, 0, 1, 3, 5, and 7. Control poly C PO-ON (40mer stabilized by 2'-O-methyl ribose modification) was also tested. A fourth group of mice did not receive any compounds (only normal saline administration). Approximately 5-15 mice were included in each group. The mice were intravenously inoculated on day 0 with HCV patient serum containing  $3.9 \times 10^3$  copies of HCV genotype 1b. Serum samples were obtained on days 0 (prior to HCV inoculation), 7, 14, 21, 28, and 35 for HCV RNA, HCV core antigen, and human albumin determination. Human albumin in the blood of the chimeric mice was measured with the Alb-II Kit (Eiken Chemical, Tokyo, Japan).

### Statistical Analysis

Data from at least triplicate experiments were averaged and expressed as means  $\pm$  standard deviations. Statistical analysis was performed using the Student *t* test or Welch *t* test. *P* values of less than .05 were considered statistically significant.

## Results

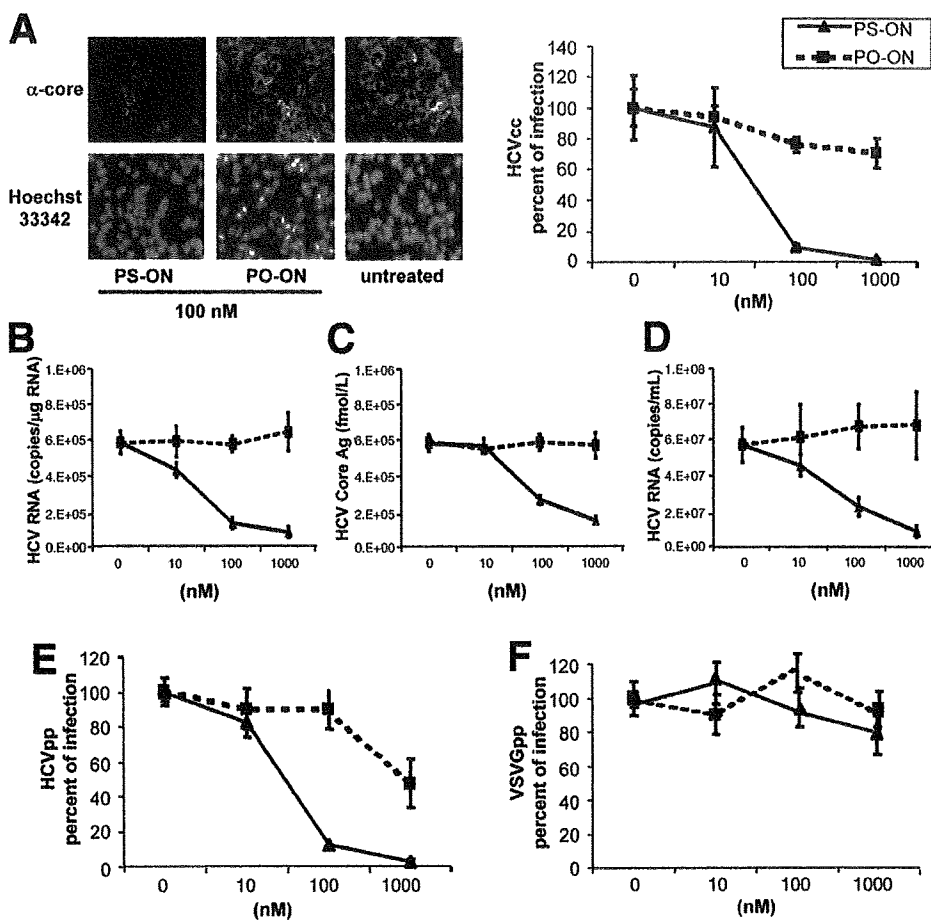
### APs Inhibit HCV Infection in a Sequence-Independent Manner

To assess whether APs can inhibit HCV infection, fully degenerate 40mer oligonucleotides that were either phosphorothioated (PS-ON) resulting in a stable amphipathic DNA polymer or that had a 2'-O-methyl modification on the ribose moiety (PO-ON) conferring stability but not altering the polyanionic nature of DNA<sup>14,23</sup> were tested. Huh7.5 cells were infected with HCVcc in the presence of either PS-ON or PO-ON. At 72 hours postinfection, HCV-infected cells were assessed by immunofluorescence assay (Figure 1A) and intracellular HCV RNA quantification (Figure 1B). HCV infection was significantly inhibited by PS-ON and not PO-ON (*P* < .05). The inhibitory effect of PS-ON was also confirmed by reduced HCV core antigen and HCV RNA levels in the culture supernatant, as compared with those of the PO-ON-treated cells (*P* < .05) (Figure 1C and D). To evaluate further the efficacy of PS-ON against viral entry, HCVpp harboring genotype 1b was used to infect Hep3B. The PS-ON blocked infection of HCVpp in a similar dose-dependent manner (Figure 1E). The PO-ON exhibited some inhibitory effect at high concentration, which could be attributed to noncytotoxic inhibition of cellular adherence by the polyanion nature of PO-ON. To assess whether the PS-ON inhibitory effect is specific for HCV, retroviral pseudovirus carrying the vesicular stomatitis virus G protein (VSVGpp) was tested in the presence of PS-ON or PO-ON. Neither PS-ON nor PO-ON had any effect on VSVGpp infection (Figure 1F). Furthermore, adenoviral infection was not inhibited by PS-ON (Supplementary Figure 1).

A series of homo- and heteropolymeric APs including poly G, A, T, C, TG AC, TC, and AG PS-ONs were tested for their inhibitory activities on HCV infection in both HCVcc and the HCVpp systems. These APs had similar inhibitory activities as the degenerate PS-ON with random sequence in the HCVcc system except for poly G and poly A (Figure 2A). Similar effects were also observed on HCV core antigen and HCV RNA levels in the culture supernatant (Figure 2B and C). In the HCVpp system, these PS-ONs also had similar inhibitory effects (Figure 2D).

### AP Inhibition of HCV Infection Is Dependent on Size and Amphipathicity

Different sizes of degenerate PS-ONs (6-, 10-, 20-, 30-, 40-, 50- and 80mer) were tested for their inhibitory



**Figure 1.** Effect of PS-ON on HCV infection. (A) Huh7.5 cells were infected with HCVcc in the presence of various concentrations of 40mer PS-ON or PO-ON (degenerate sequence). Two days after infection, infected cells were detected by immunofluorescence assay using anticore antibodies (left panel). Percentage of infection was determined by dividing the number of HCV-expressing cells in treated over the untreated cells (right panel). The intracellular HCV RNA levels (B) and HCV core Ag (C) and supernatant HCV RNA (D) levels in the culture medium were determined. Hep3B cells were infected with (E) HCVpp genotype 1b or (F) VSVGpp and treated with various concentrations of PS-ON and PO-ON, and luciferase activities were determined 2 days later. Results are shown as percentages of infection + standard deviations (SD).

activities in the HCVcc and HCVpp systems. Only PS-ONs with lengths of 40mer or greater potentially inhibited HCV infection (Figure 2E). This result was confirmed with the poly C PS-ONs (Supplementary Figure 2). To determine the requirement of amphipathicity for antiviral activity of these compounds, additional oligonucleotide analogs that had diminished hydrophilic character were prepared and include degenerate PS-ON analogs with either the base and/or the sugar removed (Supplementary Figure 3). An additional degenerate PS-ON analog containing the 2'-O-methyl ribose modification that does not affect the amphipathicity was tested. These analogs were tested for their inhibitory activities in the HCVcc and HCVpp systems. Only analogs that retained the amphipathic properties inhibited HCV infection (Figure 2F). These observations suggest that the amphipathic nature of these PS-ONs is necessary for inhibiting HCV infection.

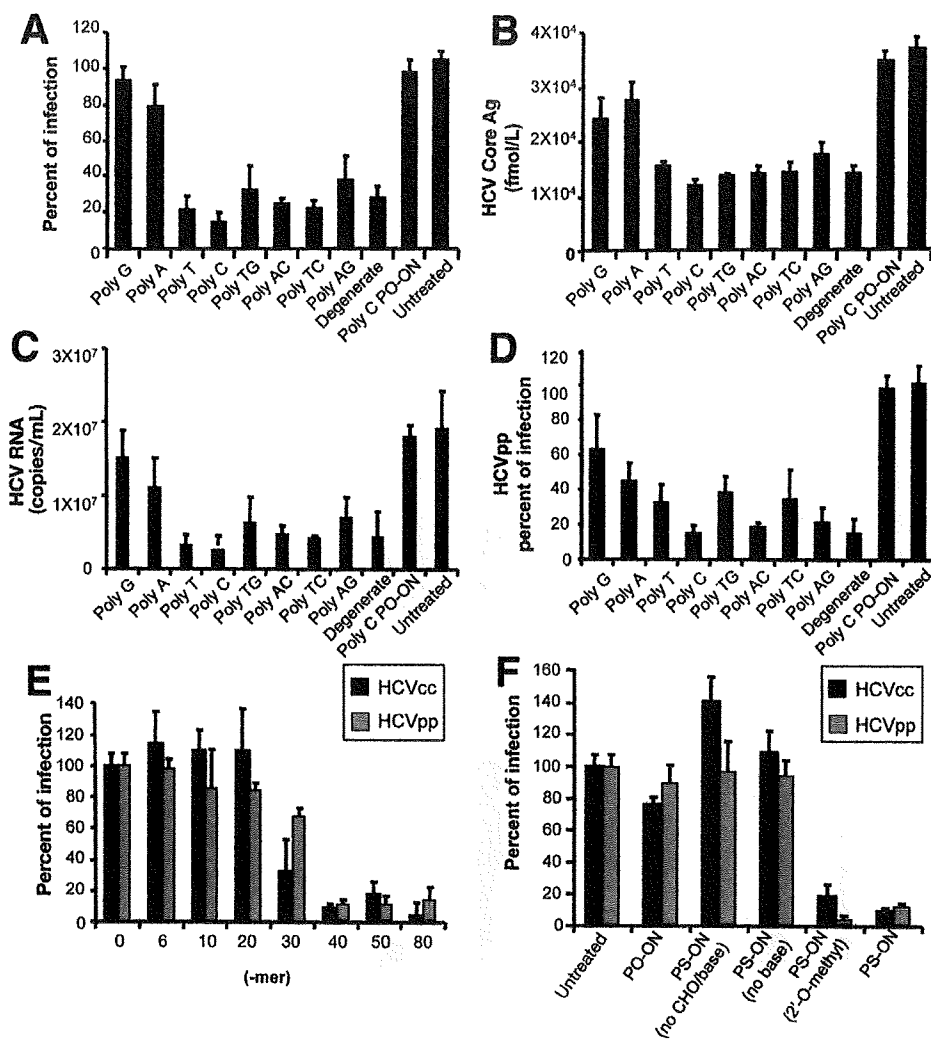
#### APs Inhibit Infection of Various Genotypes of HCV Without Affecting Replication and Cell Attachment

To study the effects of APs on various HCV genotypes, HCVpp harboring E1/E2 glycoproteins from genotypes 1a, 1b, 2a, 3a, 4a, 5a, and 6a were

tested.<sup>16</sup> Infections by all genotypes were equally blocked by the degenerate PS-ON, whereas the degenerate PO-ON had no effect (Figure 3A). Similar observation was obtained with the poly C compounds (Supplementary Figure 2D).

The degenerate PS-ON compound was tested for its effect on viral replication in the HCV replicon system, which supports viral replication without the viral entry step. Genotype 1b and 2a subgenomic replicons were tested. Subgenomic replicon RNAs containing luciferase reporter were transfected into Huh7.5 cells, and the replication efficiency was determined in the presence of the PS-ON or PO-ON control. Neither PS-ON nor PO-ON displayed any antiviral activities in both subgenomic replicon systems (Figure 3B). To eliminate the possibility that PS-ON may induce an antiviral state with increasing time of exposure to cells, the HCV replicon assay was performed after exposure to either PS-ON or PO-ON for 24–48 hours, and no difference in replication was observed (data not shown). Furthermore, Huh7.5 cells treated with PS-ON or PO-ON did not produce any detectable levels of type I interferons.

To dissect further the effect of PS-ON on viral entry, we applied the HCV-LP binding assay, which has been



**Figure 2.** Sequence-independent and size- and phosphorothioation-dependent effects of PS-ON on HCV infection. A series of 40mer PS-ONs with specific sequences including poly poly G, A, T, C, TG AC, TC, and AG were tested for their inhibitory effect on HCV infection. (A) HCVcc was inoculated with Huh7.5 cells and treated with 100 nmol/L of these homo- and heteropolymeric PS-ONs. Expression of HCV core was detected by immunofluorescence assay using anticore antibodies. (B) The HCV core Ag titers and (C) HCV RNA levels in the culture medium were determined. (D) Hep3B cells were infected with HCVpp genotype 1b and treated with these various PS-ONs at 100 nmol/L, and luciferase activities were determined 2 days later. (E) Various sizes of PS-ON (10–80mers) at 100 nmol/L were tested in the HCVcc and HCVpp systems. (F) Various structures of oligonucleotides, PS-ON analogue with phosphorothioate backbone but without the sugar or base, and PS-ON analogue with 2'-O-methyl ribose modification, were synthesized. Each 40mer oligonucleotide at 100 nmol/L was tested in the HCVcc and HCVpp systems. All results are shown as percentages of infection + SD.

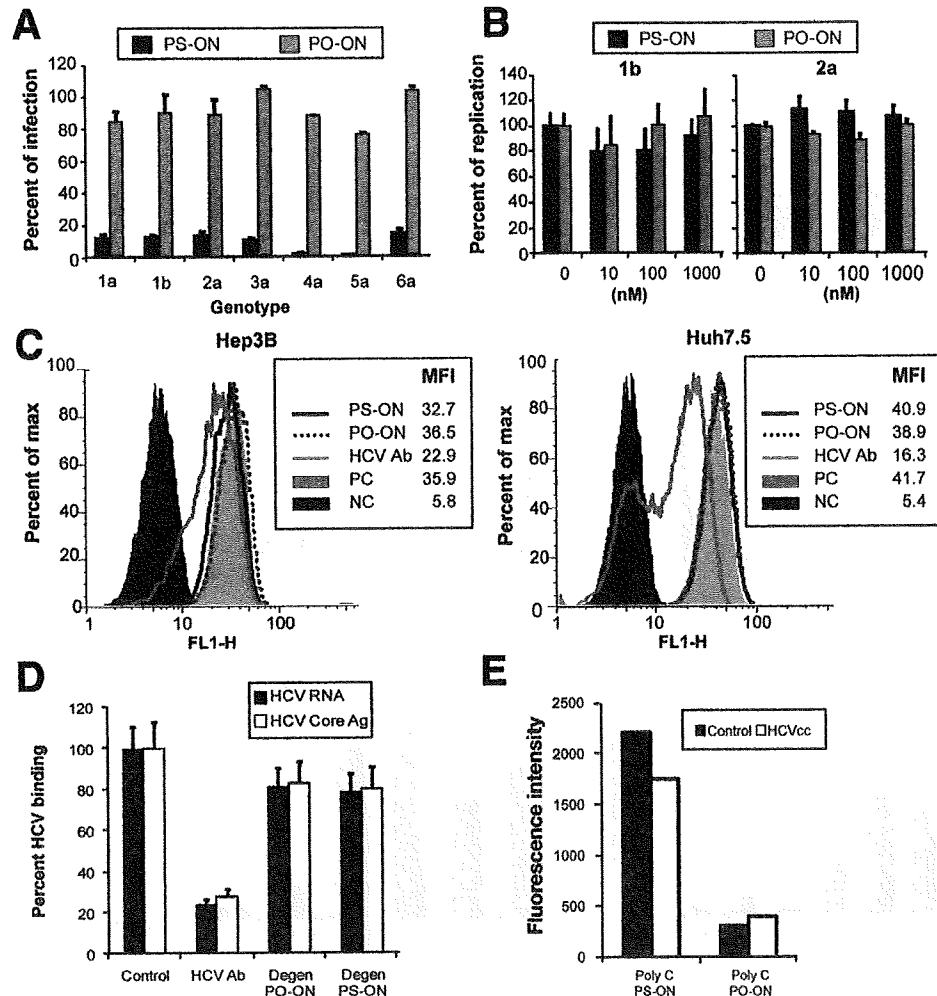
developed as a surrogate system to assess HCV binding to cells.<sup>24–26</sup> HCV-LP were incubated in the presence of PS-ON and PO-ON for 1 hour at 4°C with Huh7.5 or Hep3B cells. Under this condition, virus attaches to the cells but does not enter. HCV patients' serum containing high-level of anti-E1/E2 antibodies was included as a control. The binding was detected with FITC-labeled mouse monoclonal anti-E2 antibodies (Figure 3C). The results showed that the anti-HCV antibodies inhibited the HCV-LP binding to the cells, whereas the PS-ON and PO-ON-treated HCV-LP did not inhibit HCV-LP binding. To validate the HCV-LP binding assay, HCVcc binding to cells was performed in the presence of PS-ON, PO-ON, or HCV serum. HCV RNA bound to the cells was quantified to determine the percentage of binding. As shown in Figure 3D, HCV antibody significantly inhibited HCVcc binding to cells (~80%), whereas PS-ON and PO-ON had minor effects (<20%). These results suggest that the target of inhibition by APs is at the postbinding, cell entry step.

To address the question of whether PS-ON binds to HCV directly to inhibit HCV infection, HCV binding

assays were performed. First, in an immunoassay format using HCV-LP as a capture antigen, neither PS-ON nor PO-ON showed any significant binding to HCV-LP (Table 1). Second, sedimentation density gradient analysis did not show a preferential cosedimentation of HCVcc with PS-ON or PO-ON in comparison with the control preparation (Figure 3E), indicating that neither PS-ON nor PO-ON binds to HCVcc to any significant extent. The amount of PS-ON in the HCVcc or the control fraction was higher than that of PO-ON, probably reflecting the different physical properties of PS-ON and PO-ON. However, it is possible that low-affinity binding of HCV and PS-ON could be present and required for subsequent inhibitory action but not detected by the currently applied assays.

#### APs Inhibit Viral Internalization

To determine which entry step that APs targets, the HCVpp assay was performed in the presence of concanamycin A (25 nmol/L), degenerate PS-ON (100 nmol/L), degenerate PO-ON (100 nmol/L), or AP33+ALP98



**Figure 3.** Effects of PS-ON on infection of various HCV genotypes, HCV replication, and cell binding. (A) HCVpp harboring E1/E2 glycoproteins from genotypes 1a, 1b, 2a, 3a, 4a, 5a, and 6a were inoculated into Hep3B cells and simultaneously treated with 100 nmol/L of degenerate PS-ON and PO-ON (40mer). Luciferase activities were determined 2 days later. (B) Subgenomic RNA of genotype 1b Con1 or 2a JFH1 were transfected into Huh7.5 cells. Four hours after transfection, a set of transfected cells was harvested as a control for transfection efficacy, and the remaining cells were treated with 100 nmol/L of PS-ON and PO-ON. Cells were then harvested at 72 hours posttransfection and luciferase activities determined. The replication level was presented as the ratio of the luciferase activity of the sample at 72 hours over that of 4 hours. Percentages of replication were determined by dividing the replication level of treated over that of untreated samples. (C) Hep3B and Huh7.5 cells were incubated with 20  $\mu$ g/mL HCV-LP and 100 nmol/L PS-ON or PO-ON at 4°C for 1 hour. The cells were washed and incubated with anti-E2 ALP98 monoclonal antibody for 30 minutes followed by FITC-labeled goat anti-mouse immunoglobulin for 30 minutes at 4°C. HCV-LP binding was analyzed by flow cytometry. The *black filled peaks* are negative controls without the anti-E2 antibody. The *gray filled peaks* are positive controls showing HCV-LP binding without any compounds. The *black solid lines* and *gray dotted lines* represent treatments with PS-ON and PO-ON, respectively. The *gray solid line* represents samples in the presence of HCV serum that has been shown previously to inhibit HCV-LP binding. The mean fluorescence intensity (MFI) of each sample is shown. (D) HCVcc was incubated with Huh7.5 cells in the presence of HCV serum PS-ON or PO-ON at 4°C for 1 hour. The unbound virus was washed off, and the bound HCVcc was determined by HCV RNA quantification and HCV core Ag assay. (E) HCVcc was incubated with Cy3-labeled degenerate PS-ON or PO-ON (40mer) and subjected to iodixanol density gradient analysis as described in the online Supporting Document. Control preparation generated the same way was used for comparison. The fluorescence intensity of the fraction where infectious HCV sedimented was determined and shown.

monoclonal anti-E2 antibodies (25  $\mu$ g/mL total concentration) at 37°C. Hep3B cells were first incubated with HCVpp at 4°C to allow binding and then at 37°C with various compounds after the inoculating HCVpp was removed. Concanamycin A is known to inhibit HCV entry by preventing acidification of endosome.<sup>12</sup> As shown in Figure 4A, AP33+ALP98 anti-E2 antibodies

blocked HCV binding to the cells but had no effect on HCV entry. On the other hand, both concanamycin A and the degenerate PS-ON inhibited HCV entry.

To demonstrate that APs may inhibit HCV internalization at the fusion step, a viral fusion assay was performed with HCVpp or VSVpp as control.<sup>21</sup> Degenerate sequence and poly C PS-ONs and the control PO-ONs

**Table 1.** Lack of Binding of PS-ON to HCV-LP

	PS-ON (nmol/L) <sup>a</sup>				PO-ON (nmol/L) <sup>a</sup>				AP33 <sup>b</sup>
	0	10	100	1000	0	10	100	1000	1 $\mu$ g/mL
HCV-LP	155 + 14	105 + 9	162 + 13	116 + 10	137 + 12	15 + 10	123 + 12	157 + 13	1475 + 150
Control	147 + 13	117 + 10	120 + 11	117 + 9	111 + 10	33 + 14	100 + 8	103 + 9	153 + 16

<sup>a</sup>Cy3 labeled degenerate PS-ON and PO-ON (40mer).

<sup>b</sup>AP33 binding to HCV-LP was detected with Cy3-labeled goat anti-mouse IgG antibody.

were tested. Both PS-ON compounds showed significant inhibition of HCVpp fusion over their control PO-ON, whereas VSVGpp fusion was largely unaffected by either PS-ON or PO-ON (Figure 4B and Supplementary Figure 4). The inhibitory effect of PS-ON on fusion was evident on both the rate and maximum of fusion in the assay.

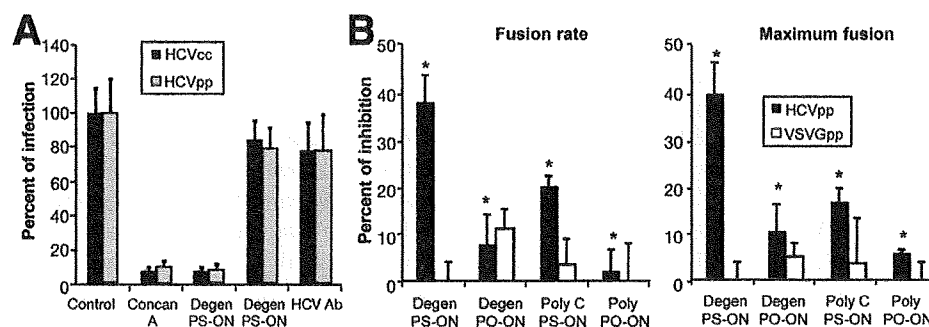
### APs Inhibit HCV Infection In Vivo

To test the efficiency of APs in vivo, sodium salts of amphipathic polymers (40mers) of poly C and poly AC and their respective PO-ON controls were prepared. Degenerate oligonucleotides were avoided because they might potentially contain CpG motifs, which could induce endogenous interferons, although in vitro testing did not reveal such a possibility. Human hepatocyte-transplanted uPA/SCID mice were treated with these compounds as described and inoculated with infectious HCV genotype 1b patient serum. In this model, the production of human albumin in serum was monitored for the engraftment index of human hepatocytes. All mice showed robust and comparable human albumin concentrations that did not change significantly during the experimental period (Figure 5). Only 1 animal in the poly C PS-ON-treated group (n = 7) and 2 in the poly AC PS-ON-treated group (n = 5) were HCV positive. The remaining mice in both groups of mice were persistently negative. All 7 mice in the poly C PO-ON-treated mice

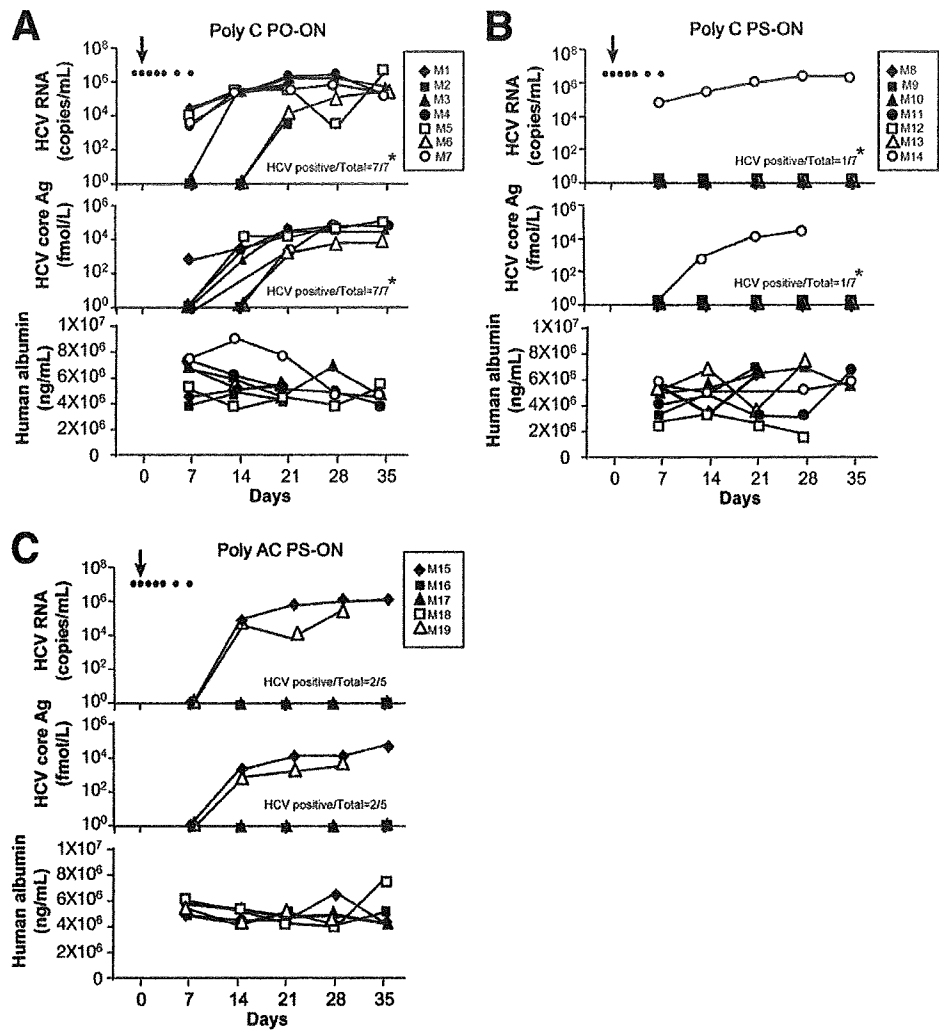
(100%) and 14 of 15 untreated mice (normal saline placebo) were HCV positive (93%). The *P* value was statistically significance between the PS-ON- and PO-ON-treated groups (*P* < .05). To rule out the possibility that these protected mice were not intrinsically resistant to HCV infection despite robust human hepatocytes engraftment, some of them were rechallenged with infectious HCV inoculum several weeks later. They all became infected, supporting the specific inhibitory effect of APs on de novo HCV infection in this in vivo model.

### Discussion

Current therapy for hepatitis C is based on peginterferon and ribavirin. However, the therapy is only effective in approximately half of the patients, and there is little option to those who fail current therapy. Recent advances in the development of small molecule inhibitors targeting the viral-encoded enzymes showed promise,<sup>27</sup> but viral resistance to these drugs is a major clinical issue because HCV is highly variable with rapid viral proliferation and low-fidelity replication. Phosphorothiate modification of oligonucleotides was initially designed to reduce enzymatic degradation. This modification also increases the hydrophobicity of the phosphodiester backbone and thus imparts an amphipathic character to the oligonucleotide polymer.<sup>28</sup> Recent studies showed that



**Figure 4.** Effects of PS-ON on HCV viral entry. (A) Hep3B cells were incubated with HCVpp at 4°C for 1 hour to bind the virus and washed to remove the unbound virus. Cells were then incubated with fresh culture medium containing 25 nmol/L concanamycin A, 100 nmol/L PS-ON, 100 nmol/L PO-ON, or 25  $\mu$ g/mL (total concentration) AP33+ALP98 monoclonal antibodies at 37°C for 16 hours. The luciferase activities were determined 24 hours later. Results are shown as percentages of infection + SD. (B) Fusion assay was performed with HCVpp or VSVGpp in the presence of PS-ON (degenerate or poly C) or the PO-ON controls. The results are expressed as mean percentages (means + SD) of inhibition of either the fusion rate at the origin of the fusion kinetics (left panel) or the maximum fusion of the curve at 500 S (right panel) relative to incubation in the absence of the compounds. The fusion curves are shown in Supplementary Figure 4. \**P* < .05 comparing the PS-ON and the corresponding PO-ON in the HCVpp fusion assay.



**Figure 5.** Effects of PS-ON on HCV infection in vivo. Human hepatocytes-transplanted uPA/SCID mice were treated intraperitoneally with 10 mg/kg of PS-ON (poly C) ( $n = 7$ ) or (poly AC) ( $n = 5$ ) on days  $-1, 0, 1, 3, 5,$  and  $7$  (indicated by dots). The corresponding control PO-ON (poly C) was also tested ( $n = 7$ ). A fourth group of mice did not receive any compounds ( $n = 15$ ). The mice were intravenously inoculated on day 0 with HCV patient serum containing  $3.9 \times 10^8$  copies of HCV genotype 1b (indicated by arrow). Serum samples were obtained on days 0 (prior to HCV inoculation), 7, 14, 21, 28, and 35 for HCV RNA and human albumin determination. HCV core antigen was also measured and showed the same results as the HCV RNA determination.

the amphipathic PS-ONs have a sequence-independent antiviral activity against HIV-1 and other viruses,<sup>14,29</sup> suggesting that these compounds may exhibit a broad-spectrum antiviral activity.

Our data showed that PS-ON blocked HCV infection in the HCVcc and HCVpp systems in a similarly dose-dependent manner, with 50% inhibitory concentration in the nanomolar range. PS-ON had no effect on infection of VSVGpp (an enveloped RNA virus with mechanism of viral entry distinct from type I and II fusion) or adenovirus (a nonenveloped DNA virus). The amphipathic nature of PS-ON is crucial for its anti-HCV property because PS-ON analogs lacking the amphipathicity are inactive. Polynucleotides are polyanions, a class of compounds that have been shown to interfere with a variety of viral infections.<sup>30,31</sup> However, our data showed clearly that the polyanionic nature is not relevant to the PS-ON inhibitory activity because the control PO-ON is not active in HCV inhibition. Furthermore, the inhibitory effect of PS-ON could not be explained by the increased stability of the phosphorothioation because the control

PO-ON has the 2'-O-methyl modification that also stabilizes the oligonucleotides.<sup>14,23</sup>

The inhibitory activity of APs is sequence independent but length dependent. The degenerate APs were equally effective as the homo- and heteropolymeric sequences, with the exception of poly A and G, which can form unique polypurine quartet structures in solution.<sup>32</sup> The minimal length of PS-ON required for potent inhibitory activities is 40mer, which appears to be the same for all active PS-ON compounds. This length-specific requirement may indicate a critical structural feature of the HCV entry process that is susceptible to these compounds. Although the degenerate PS-ON may contain CpG motif, the other hetero- and homopolymer PS-ONs tested herein are devoid of CpG motifs. The comparable antiviral activity of these compounds to the degenerate PS-ON demonstrates that the antiviral activity is not mediated by the potential CpG-mediated induction of interferon. Furthermore, Huh7.5 cells express very little or none of the cell surface toll-like receptors involved in recognition of nucleic acid-based motifs,<sup>33</sup> and we did

not observe any production of endogenous type I interferons in cells exposed to either PS-ON or PO-ON.

The inhibitory activity of PS-ON appears to target the postbinding and prereplication stage and possibly at the fusion step of HCV infection. The fusion process appears to be structurally conserved among many enveloped viruses and can be classified into types I and II.<sup>34</sup> The type I membrane fusion is exemplified by the influenza and HIV-1 via hemagglutinin and gp41, respectively. The type II fusion includes the alpha-viruses and flaviviruses.<sup>34–36</sup> It has been proposed that HCV uses a type II fusion process because of its similarity to flaviviruses.<sup>37</sup> Our recent study suggests that HCV and flaviviruses are indeed structurally similar.<sup>38</sup> It is conceivable that the fusion process of HCV may be susceptible to inhibition by the amphipathic structure of PS-ON, but further confirmation is necessary. HCV entry has been shown to occur via receptor-mediated endocytosis and is sensitive to lysosomotropic agents and inhibitors of vacuolar ATPases.<sup>39</sup> The finding that PS-ON acts at the postbinding step like concanamycin A and bafilomycin A1, which are potent inhibitors of the vacuolar ATPases, supports this hypothesis. Furthermore, all HCV genotypes appeared to be susceptible to the APs equally, suggesting that the process involved is highly conserved.

HCV entry involves multiple cellular factors, such as CD81, SR-B1, Claudin-1, heparin sulfate, DC-SIGN, and L-SIGN, and possibly LDL receptor.<sup>5–10,31</sup> CD81 and Claudin-1 have been postulated to act on the postbinding step.<sup>6,40</sup> SR-B1 is likely involved in an early viral entry step to the cells. Its interaction with apolipoproteins and cholesterol transfer property appear to be important for viral entry,<sup>41</sup> possibly at the level of membrane fusion.<sup>42</sup> The overall mechanism of HCV entry is complex and involves multiple factors and steps. The APs likely interact with 1 of these essential steps to abort HCV entry. The unique inhibitory effect of the APs on HCV infection makes it a valuable reagent to study the molecular pathway of HCV entry. The APs can also be developed as a molecular probe to image and dissect biochemically this complex process.

Our study demonstrates that APs are potent inhibitors of HCV infection. APs are equally effective against all HCV genotypes and can inhibit de novo HCV infection in the human hepatocyte-transplanted uPA/SCID mouse model. This approach has the advantage of a novel and highly conserved target mechanism that is distinct from the small molecule inhibitors being developed clinically as well as the well-established pharmacology of antisense-based nucleic acid molecules in clinical trials. The effectiveness of this class of compounds in blocking de novo HCV infection supports its value in liver transplantation to prevent reinfection, which occurs invariably and presents a major problem for the management of these patients.<sup>43</sup> So far, prophylactic reagents based on neutralizing antibodies have been disappointing in clinical trials

of liver transplantation.<sup>44</sup> Our studies illustrate the promise of this class of compounds as a potent antiviral against HCV and support its further development in the therapy of hepatitis C.

### Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at [www.gastrojournal.org](http://www.gastrojournal.org), and at doi: 10.1053/j.gastro.2009.04.048.

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#### Conflicts of interest

The authors disclose the following: J.-M.J. and A.V. are employees of REPLICor, Inc. The remaining authors disclose no conflicts.

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# Randomized Trial of High-Dose Interferon- $\alpha$ -2b Combined With Ribavirin in Patients With Chronic Hepatitis C: Correlation Between Amino Acid Substitutions in the Core/NS5A Region and Virological Response to Interferon Therapy

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The aim of this study was to compare the efficacy of high-dose interferon (IFN)- $\alpha$ -2b with standard dose of IFN- $\alpha$ -2b in combination with ribavirin (RBV) for patients with chronic hepatitis C virus (HCV) infection, and to investigate the predictive factors associated with virological response. Two hundred Japanese patients with high HCV viral load (>100 KIU/ml) were randomized to 6 or 10 mega units (MU) of 24-week IFN- $\alpha$ -2b with RBV. Predictive factors were investigated; including pretreatment amino acid (aa) sequences of the core region and the IFN-sensitive determining region (ISDR). The sustained virological response rate was not different in the two groups (24% vs. 30%) but the incidence of depression was significantly higher in the 10 MU group than 6 MU group (7% vs. 0%,  $P=0.02$ ). Younger age (<60) and HCV genotype (2a/b) were significant predictors of sustained virological response. In patients infected with genotype 1b, substitutions of core aa 70 and/or 91 were predictive for non-virological response ( $P<0.001$ ), and substitutions in the ISDR was observed frequently in virological responders. Early viral kinetics study showed that serum HCV core antigen decreased more slowly in both patients with aa 70 and/or 91 substitutions in the core and with absence of substitutions in the ISDR. In conclusion, the use of a higher dose of IFN- $\alpha$ -2b in combination with RBV did not improve virological response but resulted in higher incidence of depression. Amino acid substitutions in the core and ISDR are predictive of virological response to the therapy in patients with genotype 1b and high viral load. *J. Med. Virol.* 81:640–649, 2009.

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**KEY WORDS:** HCV; interferon; ribavirin; core region; ISDR

## INTRODUCTION

Chronic hepatitis C virus (HCV) infection is the leading cause of cirrhosis, liver failure, and hepatocellular carcinoma [Kiyosawa et al., 1990; Niederau et al., 1998]. Interferon (IFN) is an essential component of therapy for patients with chronic HCV infection. The most effective therapy available at present is the combination therapy of pegylated (PEG)-IFN and ribavirin (RBV) [Manns et al., 2001; Fried et al., 2002; Hoofnagle et al., 2003]. Among HCV genotypes, genotype 1b is the most resistant genotype to IFN therapy [Fried et al., 2002]. The limitation of use of the combination therapy for HCV infection with genotype 1b is due to the low response rate during therapy and high relapse rate after the therapy [McHutchison et al., 1998]. Several studies have evaluated the potential benefits of a larger dose of IFN with varying results [Lindsay et al., 1996; Fried et al., 2000; Ferenci et al., 2001; Hadziyannis et al., 2001; Di Marco et al., 2002; Brouwer et al., 2004]. Although treatment has been

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switched to the combination of PEG-IFN and RBV in recent years, it is important to know if a larger dose of IFN is beneficial to patients with chronic hepatitis C.

Many molecular mechanisms through which HCV evades host innate immunity have been reported to date. HCV core, E2 and NS5A proteins have been reported to inhibit the IFN signaling system [Gale et al., 1997; Taylor et al., 1999; Blindenbacher et al., 2003; Bode et al., 2003; Foy et al., 2003; Lin et al., 2006; Ciccaglione et al., 2007]. Variations of amino acid (aa) sequences in the E2 and the NS5A region have been reported to correlate with the effect of IFN therapy [Enomoto et al., 1996; Chayama et al., 1997, 2000; Polyak et al., 1998, 2000; Hashimoto et al., 1999; Puig-Basagoiti et al., 2001; Pascu et al., 2004; Gaudy et al., 2005; Brillet et al., 2007; Torres-Puente et al., 2008]. Recently, Akuta et al. [2005, 2006, 2007a, b] reported that substitution of aa 70 and/or 91 in the core region is an independent and significant predictor of non-virological response.

The aim of the present study was to evaluate the therapeutic efficacy and safety of a large dose of IFN- $\alpha$ -2b combined with RBV. For this purpose, a randomized trial was conducted to compare the therapeutic effects of high-dose (10 MU) versus standard dose (6 MU) of IFN- $\alpha$ -2b combined with RBV in patients with high HCV viral titers. The second endpoint of this study was to analyze the predictive factors associated with virological response including aa substitutions in the core region and the NS5A region.

## PATIENTS AND METHODS

### Patient Selection

Two hundred adult patients enrolled into the study. The inclusion criteria were positivity for antibody to HCV, HCV RNA levels higher than 100 KIU/ml, and the diagnosis of chronic hepatitis C was confirmed by liver biopsy. The liver biopsy specimens were evaluated as described by Desmet et al. [1994], and classified into F0 to F3. None of the patients included in this study had liver cirrhosis (F4). Other exclusion criteria included leukocytopenia (leukocyte  $<4,000/\text{mm}^3$ ) and anemia (hemoglobin concentration  $<10$  g/dl). Patients with human immunodeficiency or hepatitis B super infection, previous organ transplantation, other causes of liver disease, poorly controlled diabetes, de-compensated renal disease, pre-existing psychiatric disease, seizure disorders, cardiovascular disease, hemophilia or auto-immune type diseases were also excluded.

### Study Design

The double-blind, multi-center randomized clinical trial was conducted in 23 centers in Hiroshima city (The Hiroshima Liver Study Group). The study was approved by the Ethics Committee of Hiroshima University. Written informed consent was obtained from all participants. Eligible patients were assigned randomly into either of the two groups without further stratification using sequentially numbered cards in sealed envelopes.

Patients were randomized to treatment with combination of IFN- $\alpha$ -2b (Intron A, Shering Plough, Kenilworth, NJ) at a dose of 6 MU (Group A) or 10 MU (Group B) plus RBV (Rebetol, Shering Plough). IFN- $\alpha$ -2b was administered intramuscularly daily over the initial 2 weeks and three times weekly in the remaining 22 weeks. The dose of RBV was adjusted according to body weight (600 mg/day for  $\leq 60$  kg, 800 mg/day for  $>60$  kg). Adverse events were monitored clinically by careful interview and hematological examination throughout the study. The dosage of RBV was reduced in patients who experienced a decrease in hemoglobin concentration to  $<10$  g/dl.

Blood samples were taken 2 and 4 weeks after the beginning of therapy and every 4 weeks thereafter. Biochemical and hematological tests were performed in each center, including alanine amino transferase (ALT). Part of the serum samples were kept frozen at  $-80^\circ\text{C}$  until further analysis. Viral genotypes were determined by phylogenetic analysis after reverse transcription (RT)-polymerase chain reaction (PCR) and direct sequencing.

### Assessment of Efficacy

Serum HCV RNA was detected by nested PCR assay (Cobas Amplicor HCV test v 2.0, Roche Diagnostics, Tokyo, Japan; limit of detection, 50 IU/ml) at weeks 2, 4 and every 4 weeks during treatment and 24 weeks after the cessation of therapy. Positive samples were analyzed further by quantitative assay (Cobas Amplicor HCV monitor v 2.0, Roche Diagnostics; limit of detection, 500 IU/ml).

The primary endpoint of this study was sustained virological response, defined as undetectable serum HCV RNA by qualitative PCR test and normalization of ALT 24 weeks after the treatment. Non-virological response was applied to those patients with positive qualitative HCV RNA PCR tests in all examinations. Virological response was used to define the remaining patients who became PCR negative at least once during the treatment.

### Nucleotide Sequencing of the Core and NS5A Gene

The core aa 61–110 and NS5A aa 2209–2248 (IFN-sensitive determining region [ISDR] [Enomoto et al., 1996]) sequences were determined by direct sequencing using stored serum samples obtained just before therapy. HCV RNA was extracted from serum samples and reverse transcribed with random primers and MMLV reverse transcriptase (Takara Bio Inc., Shiga, Japan). DNA fragments were amplified by PCR using the following primers. (a) Nucleotide sequences of the core region: The first-round PCR was performed with primers CC11 (forward, 5'-GCC ATA GTG GTC TGC GGA AC-3') and e14 (reverse, 5'-GGA GCA GTC CTT CGT GAC ATG-3'), and the second-round PCR with primers CC9 (forward, 5'-GCT AGC CGA GTA GTG TT-3') and e14 (reverse) as described by Akuta et al. [2005, 2006, 2007a, b]. After denaturation at  $95^\circ\text{C}$  for 5 min, 35

cycles of amplification were set as follows; denaturation for 30 sec at 94°C, annealing of primers for 1.5 min at 57°C, and extension for 1 min at 72°C, followed by final extension at 72°C for 7 min. The second PCR was carried out with the same amplification conditions used in the first PCR, except that the second PCR primers were used instead of the first PCR primers. (b) Nucleotide sequences of ISDR in NS5A: PCR was performed with IM11 (forward, 5'-TTC CAC TAC GTG ACG GGC AT-3') and 5OA2KI (reverse, 5'-CCC GTC CAT GTG TAG GAC AT-3'). After denaturation at 98°C for 30 sec, 35 cycles of amplification were set as follows; denaturation for 10 sec at 98°C, annealing of primers for 30 sec at 66°C, and extension for 15 sec at 72°C, followed by final extension at 72°C for 5 min. The amplified PCR products were separated in a 2% agarose gel and purified by GENE-CLEAN II kit (Q-Bio Gene, Carlsbad, CA). Nucleotide sequences were determined using Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan). Nucleotide and aa sequences were compared with the nucleotide sequences of genotype 1b HCV-J (Gene Bank accession number; D90208) [Kato et al., 1990].

### Quantitation of HCV Core Antigen

HCV core antigen levels were measured using stored serum samples just before and 4 weeks after the start of the therapy as described previously [Aoyagi et al., 1999].

### Statistical Analysis

The baseline characteristics of the patients in the two groups were compared and the differences were

assessed by Chi-square test with Yate's correction and Mann-Whitney *U*-test. To assess the sustained virological response rates, an intention-to-treat (ITT) analysis and a per-protocol (PP) analysis were conducted. The response rates and substitutions in the core region and the ISDR were compared by Fisher's exact test. All *P* values reported are two-sided and those less than 0.05 were considered significant. To determine the predictors of sustained virological and non-virological responses, univariate and multivariate logistic regression analyses were carried out. Potential predictive factors included the following variables: age, sex, alcohol consumption, past history of IFN monotherapy, body mass index, ALT, hemoglobin, platelets, HCV RNA level, genotype, liver histology, total RBV dose (adjusted for body weight [mg/kg]) and total dose of IFN- $\alpha$ -2b. The odds ratio and 95% confidence intervals (95% CI) were also calculated. Variables with statistical significance ( $P < 0.05$ ) or marginal significance ( $P < 0.10$ ) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. Statistical analyses were performed using the SPSS software (SPSS, Inc., Chicago, IL).

## RESULTS

### Patient Demographics

Patient enrollment started in January 2002, and the trial ended in March 2005. The disposition of patients throughout the trial is shown in Figure 1. A total of 200 patients were randomized to treatment, and 198 patients met the eligibility criteria and underwent

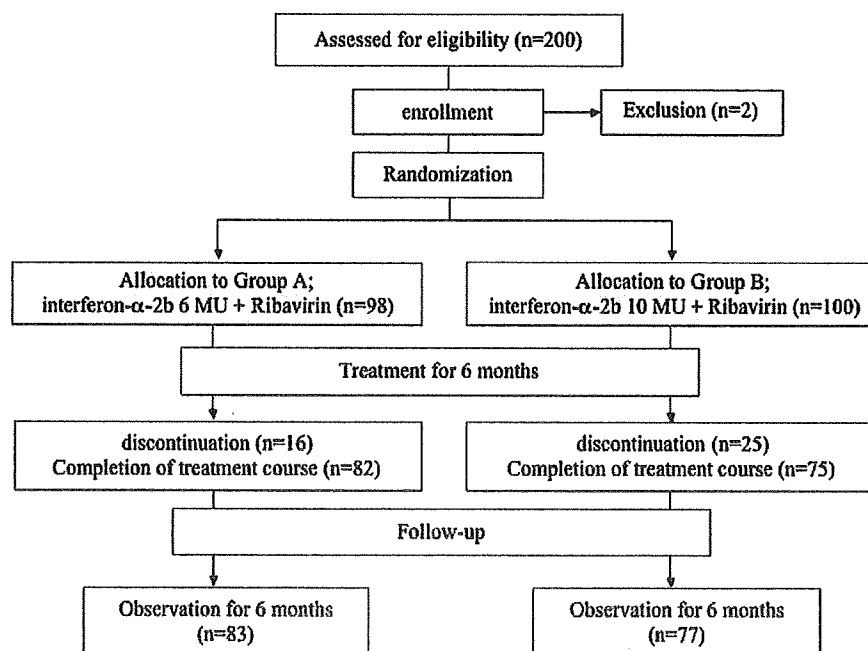


Fig. 1. Flow chart of number of patients throughout the trial. A total of 200 patients were included in this study. One hundred ninety-eight patients met the eligibility criteria and they underwent randomization, 98 patients in Group A and 100 patients in Group B.

TABLE I. Baseline Characteristics of the Patients

Characteristic	Group A (n = 98)	Group B (n = 100)	P
Age (years) <sup>a</sup>	55 ± 10.3	55 ± 11.0	0.43
Male sex (%)	63	75	0.07
Alcohol consumption (%) <sup>b</sup>	23	20	0.61
Past history of IFN monotherapy (%)	33	35	0.72
Body-mass index (kg/m <sup>2</sup> ) <sup>a</sup>	23.3 ± 2.9	24.2 ± 3.6	0.05
ALT (IU/L) <sup>a</sup>	79.2 ± 45.3	109.4 ± 111.2	0.31
Hemoglobin (g/dl) <sup>a</sup>	14.2 ± 1.4	14.5 ± 1.2	0.02
Platelets (×10 <sup>4</sup> /mm <sup>3</sup> ) <sup>a</sup>	14.8 ± 4.8	16.5 ± 5.0	<0.05
HCV RNA (KIU/ml) (%)			
100–850	49	47	
≥850	51	53	0.80
Genotype (%)			
1b	82	72	
2a/2b	17	28	0.32
3a/3b	1	0	
Liver histology <sup>a,c</sup>	2.0 ± 0.84	1.8 ± 0.82	0.05

ALT, alanine aminotransferase.

<sup>a</sup>Values are mean ± SD.

<sup>b</sup>Percentage of patients who consumed alcohol at >30 g/day.

<sup>c</sup>Liver fibrosis was scored 0 (F0), no fibrosis; 1 (F1), periportal expansion; 2 (F2), portoportal septa; 3 (F3), portocentral linkage or bridging fibrosis.

randomization. Ninety-eight patients were assigned to Group A and 100 patients to Group B. Patients were observed for 24 weeks after the treatment. Sixteen patients of Group A and 25 patients of Group B discontinued the treatment because of adverse events. Table I lists the baseline characteristics of the patients. Hemoglobin concentrations and platelet counts were higher in group B patients. The other parameters were similar between the two groups.

#### Overall Sustained Virological Response

The effect of therapy in the two groups is summarized in Table II. The sustained virological response rate was lower significantly in patients of group B with genotype 2a/b relative to those of group A (ITT analysis). This reflects the fact that a larger number of patients dropped out from the protocol because of the adverse effects (1 [6%] of 16 in group A and 10 [43%] of 23 in group B,  $P=0.02$ ). All patients who stopped treatment did not achieve sustained virological response. Patients with genotype 1b had a lower sustained virological response rate than those with genotype 2a/b (33/124 [27%] vs. 26/39 [67%],  $P < 0.01$ ).

TABLE II. Rates of Sustained Virological Response According to Adherence

Genotype	Group A	Group B	P
1b	n = 68	n = 56	
ITT	16/68 (24%)	17/56 (30%)	0.39
PP	16/53 (30%)	17/41 (41%)	0.25
2a/b	n = 16	n = 23	
ITT	15/16 (94%)	11/23 (48%)	0.005
PP	15/15 (100%)	11/13 (85%)	0.21

ITT, intention to treatment analysis; PP, per protocol analysis; IFN, interferon; RBV, ribavirin.

#### Dose Reduction or Discontinuation and Adverse Events

Table III summarizes the laboratory abnormalities and the dose reduction and discontinuation of IFN- $\alpha$ -2b and RBV due to adverse events. The overall discontinuation rate was 16% for group A and 25% for group B (not significant). The most frequent adverse event associated with dose reduction was anemia. A larger number of patients of group B developed depression ( $P=0.02$ ).

#### Predictive Factors Associated With Sustained Virological Response

Univariate analysis identified three parameters that correlated with sustained virological response: age (<60 years,  $P=0.007$ ); genotype (2a/b,  $P < 0.001$ ); and platelet count ( $>15 \times 10^4/\text{mm}^3$ ,  $P=0.01$ ). Multivariate analysis including the above variables identified two parameters that independently predicted sustained virological response: age ( $P=0.02$ ) and genotype ( $P < 0.001$ ) (Table IV).

TABLE III. Dose Reduction or Discontinuation and Adverse Events

	Group A (n = 98) %	Group B (n = 100) %	P
Discontinuation	16 (16)	25 (25)	0.13
Dose reduction or discontinuation of			
IFN	20 (20)	41 (41)	0.002
RBV	36 (35)	50 (50)	0.04
IFN and/or RBV	37 (36)	55 (55)	0.01
Depression	0 (0)	7 (7)	0.02

IFN, interferon; RBV, ribavirin.



TABLE V. Amino Acid Substitutions in the Core Region in Non-Virologic Responders and Virological Responders in 93 Patients With HCV Genotype 1b

Presence of substitution site	Non-virological response (n = 11) % (n)	Virological response (n = 82) % (n)	P
aa 70	64 (7)	23 (19)	0.01
aa 75	73 (8)	45 (37)	0.11
aa 91	82 (9)	30 (25)	0.001
aa 106	27 (3)	31 (26)	1.0
aa 110	18 (2)	12 (10)	62
aa 70 and 91	45 (5)	10 (8)	0.006
aa 70 and/or 91	100 (11)	44 (36)	<0.001

aa, amino acid.

substitutions of aa. Among aa substitutions, only substitutions of aa 70 and 91 were associated with non-virological response. All non-virological responders had aa substitutions at 70 or 91, or both substitutions. In contrast, only 36 of 82 (44%) virological responders had these substitutions ( $P < 0.001$ , Table V). In contrast to non-virological response, these substitutions were not predictive for sustained virological response ( $P = 0.11-0.82$ ).

Next, the effect of substitutions of aa 70 and 91 in the core region on early viral kinetics was analyzed by dividing patients into four groups according to the pattern of aa substitutions. As shown in Figure 3, the most rapid decrease in core antigen was noted in patients where both aa 70 and 91 were wild-type (double-wild). In contrast, the poorest reduction was

noted in patients with both of aa 70 and 90 substitutions (double-mutant). Patients with either of the two aa substitutions (mutant/wild or wild/mutant) showed decrease in between the above two groups. HCV core antigen decreased below the detectable limit (20 fmol/L) at week 4 in 37 of 40 (93%) patients who had neither aa 70 nor aa 90 substitutions. In contrast, it decreased below the detectable limit in only 5 of 12 patients (42%) who had both aa 70 and 91 substitutions ( $P = 0.031$ ).

#### Analysis of Nucleotide Sequence of the NS5A Gene

The aa sequences of ISDR in the NS5A gene were determined in 40 patients where PCR for this region was positive. Seventeen of 40 patients had no aa

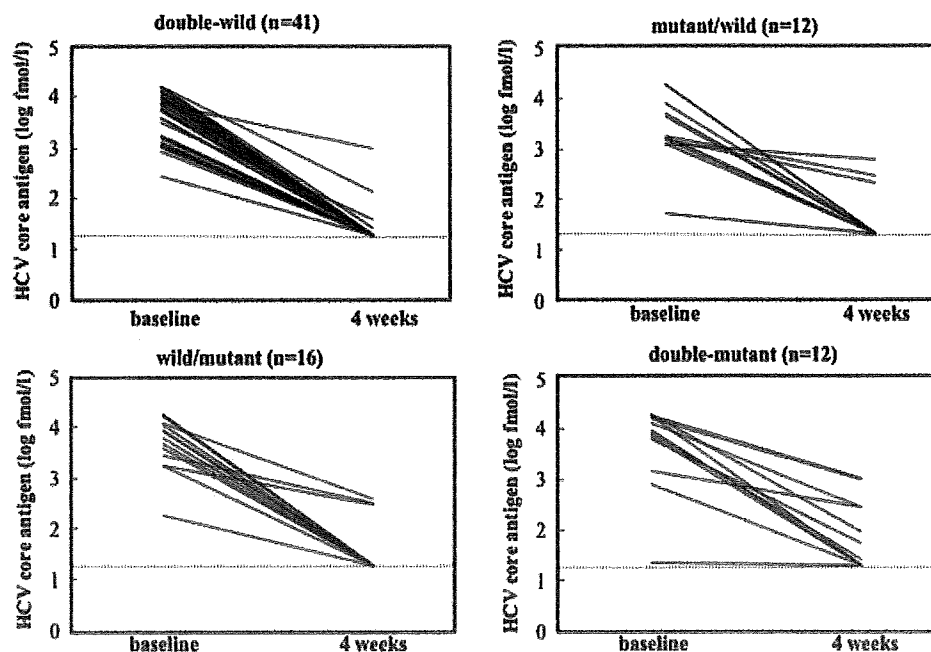


Fig. 3. Reduction of amount of HCV core antigen based on the presence of substitutions at amino acid 70 or 91. Eighty-one patients infected with hepatitis C virus were treated with combination therapy. Serum HCV core antigen was measured before treatment (baseline) and at week 4. The response was divided into four patterns based on the presence of substitution(s) at aa 70 and/or 91. Double-wild; no

substitution, neither at aa 70 nor aa 91, mutant/wild; substitution only at aa 70, wild/mutant; substitution only at aa 91, double-mutant; substitutions at both aa 70 and 91. The fixed-quantity bottom value of HCV core antigen was 20 fmol/L calculated 1.3 in log, indicated by the dotted lines.

TABLE VI. Amino Acid Substitutions in the IFN-Sensitive Determining Region (ISDR) in Non-Virological Responders and Virological Responders in 40 Patients With HCV Genotype 1b

ISDR <sup>a</sup>	Non-virological response (n = 8) % (n)	Virological response (n = 32) % (n)	P
Wild-type (n = 17)	36 (6)	64 (11)	0.012
Mutant-type (n = 23)	9 (2)	91 (21)	

aa, amino acid.

<sup>a</sup>Absence of amino acid substitutions was evaluated as wild-type, and presence of one or more amino acid substitutions as mutant-type.

substitutions in ISDR (wild-type), while the remaining 23 patients had one or more substitutions (mutant-type). The relationship between aa substitutions of ISDR and effects of treatment was analyzed. The existence of aa substitution in the ISDR was not predictive for sustained virological response ( $P = 0.137$ ), however, such substitution was observed frequently in virological responders compared to non-virological responders (66% vs. 25%,  $P = 0.012$ ) (Table VI). The use of a different categorization based on the number of substitutions in the ISDR (0/1 vs.  $\geq 2$ ) yielded similar results, that is, not predictive for sustained viral response but predictive for virological responders (data not shown).

HCV core antigen decreased more rapidly in patients with ISDR mutant-type compared to those with wild-type (Fig. 4). HCV core antigen decreased below the detectable limit at week 4 in only 6 of 17 (35%) patients with wild-type. In contrast, it decreased below the detectable limit in 19 of 23 (83%) in patients with ISDR mutant-type ( $P = 0.006$ ).

#### Predictive Factors Associated With Sustained Virological Response and Non-Virological Response in Patients With Genotype 1b

Finally, the predictive factors associated with sustained virological response and non-virological response were analyzed in patients with genotype 1b, including aa substitutions in the core region and ISDR. Univariate

analysis showed two parameters correlated with sustained virological response: age ( $< 60$  years,  $P = 0.004$ ) and presence of aa substitutions in the core (aa 70 and/or 91,  $P = 0.04$ ). However, multivariate analysis, including the above variables, identified no parameters that influenced sustained virological response independently (age,  $P = 0.89$ ; core,  $P = 0.07$ ). Univariate analysis showed two parameters correlated with non-virological response: age ( $< 65$  years,  $P = 0.02$ ) and aa substitutions in the core (double-mutant,  $P = 0.01$ ). Multivariate analysis including the above variables identified aa substitutions in the core as an independent factor that influenced non-virological response (age,  $P = 0.40$ ; core,  $P = 0.03$ ) (Table VII).

#### DISCUSSION

Treatment of patients with chronic HCV infection had improved by the advent of PEG-IFN and RBV combination therapy. However, a substantial number of patients do not respond to the combination therapy [Taliani et al., 2006]. Several studies described attempts to improve the sustained virological response rate in such patients. Recent trials showed that a longer treatment period results in a higher sustained virological response rate [Berg et al., 2006; Sánchez-Tapias et al., 2006]. However, there are no conclusive studies that compared a larger dose of IFN with standard dose. Although the treatment had shifted in recent years to PEG-IFN and

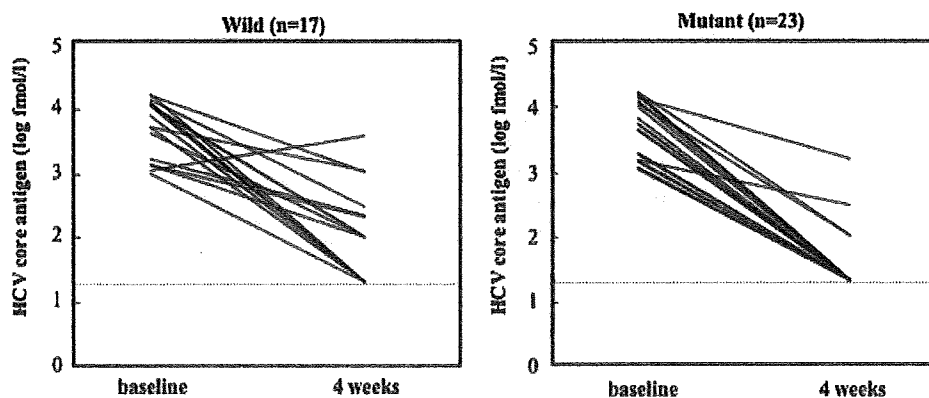


Fig. 4. Reduction of amount of HCV core antigen based on the presence of substitutions in the ISDR. Sixty-five patients infected with hepatitis C virus were treated with combination therapy. Serum HCV core antigen was measured before treatment (baseline) and at week 4. Patients were divided into two groups based on the presence of amino acid substitution(s) in the ISDR. Wild-type; absence of substitutions, mutant-type; presence of one or more substitutions. The fixed-quantity bottom value of HCV core antigen was 20 fmol/L calculated 1.3 in log, indicated by the dotted lines.