24 Henry M, Guétard D, Suspène R, Rusniok C, Wain-Hobson S, Vartanian JP. Genetic editing of HBV DNA by monodomain human APOBEC3 cytidine deaminases and the recombinant nature of APOBEC3G. *PLoS ONE* 2009; **4**: e4277.
25 Kidd JM, Newman TL, Tuzun E, Kaul R, Eichler EE. Population stratification of a common APOBEC gene deletion polymorphism. *PLoS Genet* 2007; **3**: e63.
26 Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology* 1996; **24**: 289–93.
27 Price PM, Banerjee R, Jeffrey AM, Acs G. The mechanism of inhibition of hepatitis B virus replication by the carbocyclic analog of 2′-deoxyguanosine. *Hepatology* 1992; **16**: 8–12.
28 Ohnishi Y, Tanaka T, Ozaki K, Yamada R, Suzuki H, Nakamura Y. A high-throughput SNP typing system for genome-wide association studies. *J Hum Genet* 2001; **46**: 471–7.
29 Kobayashi M, Takaori-Kondo A, Shindo K, Abudu A, Fukunaga K, Uchiyama T. APOBEC3G targets specific virus species. *J Virol* 2004; **78**: 8238–44.
30 Tsuge M, Hiraga N, Takaishi H *et al.* Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. *Hepatology* 2005; **42**: 1046–54.
31 Noguchi C, Hiraga N, Mori N *et al.* Dual effect of APOBEC3G on hepatitis B virus. *J Gen Virol* 2007; **88**: 432–40.
32 Nielsen DM, Ehm MG, Weir BS. Detecting marker-disease association by testing for Hardy-Weinberg disequilibrium at a marker locus. *Am J Hum Genet* 1998; **63**: 1531–40.
33 Noguchi C, Imamura M, Tsuge M *et al.* G to A hypermutation in hepatitis B virus and clinical course of patients with chronic hepatitis B. *J Inf Dis* 2009; **199**: 1599–607.

G-to-A Hypermutation in Hepatitis B Virus (HBV) and Clinical Course of Patients with Chronic HBV Infection

Chiemi Noguchi,[1,2] Michio Imamura,[1,2] Masataka Tsuge,[1,2] Nobuhiko Hiraga,[1,2] Nami Mori,[1,2] Daiki Miki,[1,2] Takashi Kimura,[1,2] Shoichi Takahashi,[1,2] Yoshifumi Fujimoto,[1,2] Hidenori Ochi,[2,3] Hiromi Abe,[1,3] Toshiro Maekawa,[3] Chise Tateno,[2,4] Katsutoshi Yoshizato,[2,4] and Kazuaki Chayama[1,2,3]

[1]Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, and [2]Liver Research Project Center, Hiroshima University, Hiroshima, [3]Laboratory for Liver Diseases, Single-Nucleotide Polymorphism Research Center, the Institute of Physical and Chemical Research, Yokohama, and [4]PhoenixBio, Higashihiroshima, Japan

Background. The apolipoprotein B messenger RNA editing enzyme, catalytic polypeptide–like family of cytidine deaminases induce G-to-A hypermutation in hepatitis B virus (HBV) genomes and play a role in innate antiviral immunity. The clinical relevance of this protein family is unknown.

Methods. We analyzed 33 instances in which 17 patients with chronic HBV infection experienced >2 increases of >100 IU/L in alanine aminotransferase (ALT) level; we used a quantitative differential DNA denaturation polymerase chain reaction assay to quantify the hypermutated HBV genomes observed during 21 of these 33 increases in ALT level.

Results. Of the 9 increases in ALT level that involved a >5-fold increase (relative to basal levels) in the number of hypermutated genomes observed, 8 were associated with a >2-log reduction in plasma HBV DNA level. In contrast, a corresponding decrease in plasma HBV DNA level was observed for only 1 of the 12 increases in ALT level that did not involve an increase in the number of hypermutated genomes ($P < .001$). Hepatitis B e antigen clearance was often observed in patients who experienced an increase in the number of hypermutated genomes. Interferon treatment induced hypermutation in HBV genomes in an animal model. However, there was no apparent increase in the number of hypermutated genomes among the majority of patients who received interferon therapy, probably because the number of hypermutated genomes had already increased prior to the initiation of therapy.

Conclusion. Our results suggest that a marked increase in the number of hypermutated genomes represents a strong immunological host response against the virus and is predictive of hepatitis B e antigen clearance and plasma HBV DNA level reduction.

Despite the availability of safe and effective vaccines for >2 decades, hepatitis B virus (HBV) infection is still a global health problem. Worldwide, >2 billion people are infected with HBV, and chronic HBV infection affects ~400 million people [1, 2]. It is estimated that >500,000 people die annually because of cirrhosis and/or hepatocellular carcinoma due to HBV infection [3].

Recent reports have shown that cellular cytosine deaminase (apolipoprotein B messenger RNA [mRNA] editing enzyme, catalytic polypeptide–like 3G [APOBEC3G]), packaged in human immunodeficiency virus type 1 (HIV-1), induces G-to-A hypermutation to a nascent reverse transcript of HIV-1 and reduces the infectivity of HIV, thus contributing in part to innate antiviral activity [4–8]. HIV-1 overcomes this innate defense barrier in T cells with HIV virion infectivity factor, a protein that specifically targets APOBEC3G to proteasomal degradation [9–12]. HIV-1 can infect resting CD4 T cells in lymphoid tissues but not those circulating in peripheral blood [13–16]. Resting CD4 T cells in peripheral blood are protected from HIV infection through the action of the deaminase-active

Received 30 August 2008; accepted 6 November 2008; electronically published 28 April 2009.

Potential conflicts of interest: none reported.

Financial support: Ministry of Education, Sports, Culture and Technology and Ministry of Health, Labor and Welfare (Grants-in-Aid for scientific research and development).

Reprints or correspondence: Kazuaki Chayama, MD, Dept. of Medical and Molecular Science, Div. of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan (chayama@hiroshima-u.ac.jp).

The Journal of Infectious Diseases 2009; 199:1599–607

0022-1899/2009/19911-0007$15.00
DOI: 10.1086/598951

Table 1. Clinical profiles of 17 patients with chronic hepatitis B virus (HBV) infection who experienced >2 increases of >100 IU/L in alanine aminotransferase (ALT) level.

Patient	Sex	Age, years	ALT level, IU/L		Plasma HBV DNA level, log copies/mL	HBV serum marker status[a]		HBV subtype	Histologic result[b]	Receipt of IFN treatment
			Minimum	Maximum		HBeAg	HBeAb			
1	M	50	26	2000	8.1	+	−	C	F2, A2	Yes
2	M	31	22	230	8.2	+	−	C	F3, A2	Yes
3	F	23	14	313	8.7	+	−	C	F2, A2	Yes
4	M	22	16	846	6.9	+	−	C	F2, A1	Yes
5	F	42	10	100	7.8	+	−	C	L	No
6	F	33	21	748	8.8	+	−	C	F2, A3	Yes
7	M	23	22	339	8.4	+	−	C	L	Yes
8	F	54	22	108	6.7	−	+	C	F2, A2	No
9	M	44	17	512	9.5	+	−	C	F2, A3	No
10	M	27	39	115	8.8	+	−	C	F1, A1	Yes
11	M	36	16	452	3.8	+	−	C	F4, A3	Yes
12	M	20	21	1295	7.2	+	−	C	F2, A2	No
13	M	36	24	481	5.7	−	+	C	F2, A2	Yes
14	M	22	20	696	5.9	+	−	C	F1, A1	Yes
15	F	24	14	1544	7.7	+	−	C	F2, A2	Yes
16	M	35	10	1618	4.7	+	−	C	F2, A1	Yes
17	M	30	21	1655	6.7	+	−	C	L	Yes

NOTE. HBeAg, HBV e antigen; HBeAb, antibody against HBV e antigen; IFN, interferon; L, liver cirrhosis.

[a] Before increase in ALT level.

[b] Histologic evaluation of chronic hepatitis by use of the scoring system of Desmet et al. [29].

APOBEC3G [17]. Recent reports have shown that interferon (IFN)–α is a potent inducer of APOBEC3G [18–21]. It has also been reported that some of the HIV restriction exerted by APOBEC3G may be independent of its cytidine deaminase activity [17, 22–24].

We and others have reported the presence of small numbers of hypermutated genomes in serum samples obtained from HBV-infected patients [25–27]. Studies using HepG2 cell lines and primary human hepatocytes showed that such hypermutation is induced by the cytidine deaminase activity of the APOBEC family of proteins [27]. In our previous study, IFN induced little hypermutation in the HBV genome [27]. However, after extensive investigation supported by development of a quantitative analysis of hypermutation, we showed that both IFN-α and IFN-γ actually increase transcription of APOBEC3G mRNA in HepG2 cell lines and induce an increase in the number of hypermutated genomes [28]. We also showed that APOBEC3G induces hypermutation in HBV and reduces HBV replication levels in the absence of the deaminase activity. Thus, APOBEC3G has dual antiviral actions against HBV and is thought to be part of the host defense mechanisms, as has been shown for HIV infection. Although it is assumed that APOBEC3G is important in the host anti-HBV defense system, little is known about the clinical importance of this enzyme, because there are no methods available for the precise quantification of small amounts of hypermutated genomes.

Using a method that can measure small amounts of hypermutated genomes (differential DNA denaturation polymerase chain reaction [3D-PCR] combined with TaqMan PCR [28]), we analyzed fluctuations in the number of hypermutated genomes observed in patients with chronic HBV infection who experienced increased alanine aminotransferase (ALT) levels. The study group included patients who received IFN treatment and patients who did not.

METHODS

Patients. From 2002 through 2006 at Hiroshima University Hospital (Hiroshima, Japan), there were 17 consecutive patients with chronic hepatitis B who experienced >2 increases of >100 IU/L in ALT level and for whom stored serum samples were available. These 17 patients were enrolled in this study, among whom 33 such increases in ALT level were observed. Thirteen of 17 patients received IFN treatment, usually during an increase in ALT level. The clinical profiles of these 17 patients are shown in table 1. Written informed consent was obtained from all patients, and the study was approved by the Hiroshima University Ethics Committee.

HBV markers. Hepatitis B e antigen and antibody against e antigen were quantified by use of enzyme immunoassay kits (Abbott Diagnostics). HBV DNA was measured by use of real-time PCR performed with the 7300 Real-Time PCR System (Applied Biosystems), in accordance with the manufacturer's instructions. The primers used for amplification were 5'-TT-

TGGGCATGGACATTGAC-3' (nt 1893–1912; nucleotide numbers are those of HBV subtype C as reported by Norder et al. [30]) and 5'-GGTGAACAATGTTCCGGAGAC-3' (nt 2029–2049). For real-time PCR, we used 25 μL of SYBR Green PCR Master Mix (Applied Biosystems) with 1 μL of the DNA solution and 200 nmol/L of each primer. The amplification conditions were as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of amplification (denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min). The lower detection limit of this assay was 10^3 copies/mL.

Extraction of HBV DNA and quantitative analysis of hypermutated genomes. HBV DNA was extracted from 100-μL serum samples by use of the SMITEST DNA Extraction Kit (Genome Science Laboratories) and dissolved in 20 μL of water. Hypermutated genomes were quantified by use of TaqMan 3D-PCR performed with the 7300 Real-Time PCR System (Applied Biosystems); we used a procedure described elsewhere [28], with slight modifications. In brief, the HBV DNA fragments were amplified by use of 3D-PCR in which the denaturation temperature was set lower than usual so that only G-to-A hypermutated genomes would be amplified. The amplification conditions were as follows: activation at 95°C for 10 min; followed by initial denaturation at 89°C for 20 min, to allow nonhypermutated genomes reanneal; and 45 cycles of amplification (denaturation at 89°C for 20 s, annealing at 50°C for 30 s, and extension at 62°C for 90 s). TaqMan PCR was performed using the following primers: 5'-ACTTCAACCCCAACAMRRATCA-3' (nt 2978–2999) and 5'-AGAGYTTGKTGGAATGTKGTGGA-3' (nt 24–1), where M is A or C, R is G or A, Y is T or C, and K is G or T. The probe was a 6-carboxyfluorescein (FAM)–labeled MGB probe, 5'-(FAM)-TTAGAGGTGGAGAGATGG-(MGB)-3' (nt 3184–3167). The detection limit of hypermutated genomes was 10^2 copies/mL, and nonhypermutated genomes were not amplified by 3D-PCR [28]. The reproducibility of the assay was quite high (as indicated by the small standard deviation relative to the results of the quantitative PCR control reaction), as reported in our previous study [28].

Cell culture and transfection. HepG2 cell lines were grown in Dulbecco's modified Eagle medium supplemented with 10% (vol/vol) fetal calf serum at 37°C in 5% CO_2. Cells were seeded to semiconfluence in 6-well tissue culture plates and transfected with the plasmid pTRE-HB-wt, which contained 1.4–genome length wild-type HBV genomes [31], by calcium phosphate precipitation. Seventy-two hours after transfection, the supernatant was collected for HBV DNA quantification by real-time PCR and for quantitative analysis of G-to-A hypermutated genomes [28]. The remaining supernatant was stored at −80°C for infection experiments using human hepatocyte–chimeric mice.

Quantitative analysis of G-to-A hypermutated genomes with human hepatocyte–chimeric mice. A human hepatocyte–chimeric mouse model was developed, as described previously [32], and used in infection and IFN-treatment experiments. The human hepatocytes progressively repopulated the murine host liver and were susceptible to HBV produced in cultured cell lines [31]. All animal protocols were in accordance with the guidelines of the local animal experimentation committee. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University. Hepatocyte-chimeric mice were inoculated with 500 μL of the supernatant produced by transiently transfected cell lines. After confirmation of high-level HBV viremia, the mice were treated with 7000 IU/g/day of IFN-α, injected intramuscularly, for 14 days (the IFN-α was a gift from Hayashibara Biochemical Labs in Okayama, Japan). Human serum albumin in mouse serum was measured with the Human Albumin ELISA Quantitation Kit (Bethyl Laboratories), used in accordance with the manufacturer's instructions.

Statistical analysis. Differences between clinical groups with respect to HBV DNA and e antigen levels were examined for statistical significance, using the Mann-Whitney *U* test. A *P* value $<.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed with StatView (version 5.0; SAS Institute).

RESULTS

Clinical course of disease in patients with increased ALT levels and fluctuations in the number of hypermutated genomes. Figure 1*A*–1*D* shows clinical courses for 4 representative patients (patients 1–4 in Table 1) with chronic HBV infection who experienced increases in ALT level. We observed marked decreases in HBV DNA level in association with marked increases in hypermutated genomes (figure 1*A*–1*C*, black arrows). In contrast, there was no apparent reduction in HBV level in the absence of an increase in hypermutated genomes (1*A*–1*D*, white arrows). We also analyzed the effect of IFN therapy on the number of hypermutated genomes. In some patients, we observed an increase in the number of hypermutated genomes during IFN therapy (figure 1*B* and 1*C*) as well as a marked increase in the number of hypermutated genomes and a reduction of the virus accompanied by an increase in ALT level just after cessation of IFN therapy (1*A*–1*C*, black arrows). However, in some patients, such as patient 1 (figure 1*A*), we observed no apparent increase in the number of hypermutated genomes in response to IFN therapy. However, the number of hypermutated genomes observed in samples from this patient obtained just before the initiation of IFN therapy ($996/10^6$ genomes) was already higher than the baseline level ($157/10^6$ genomes). Samples from patient 4 (figure 1*D*) showed an increase in the number of hypermutated genomes during IFN therapy ($1907/10^6$ genomes), though this is less than the increase observed during natural exacerbation ($12{,}404/10^6$ genomes). In fact, there was no significant difference between IFN-treated patients and untreated patients with respect to the number of hypermutated genomes observed (data not shown). These results suggest that the host's antiviral immunity level was higher at baseline than it was after

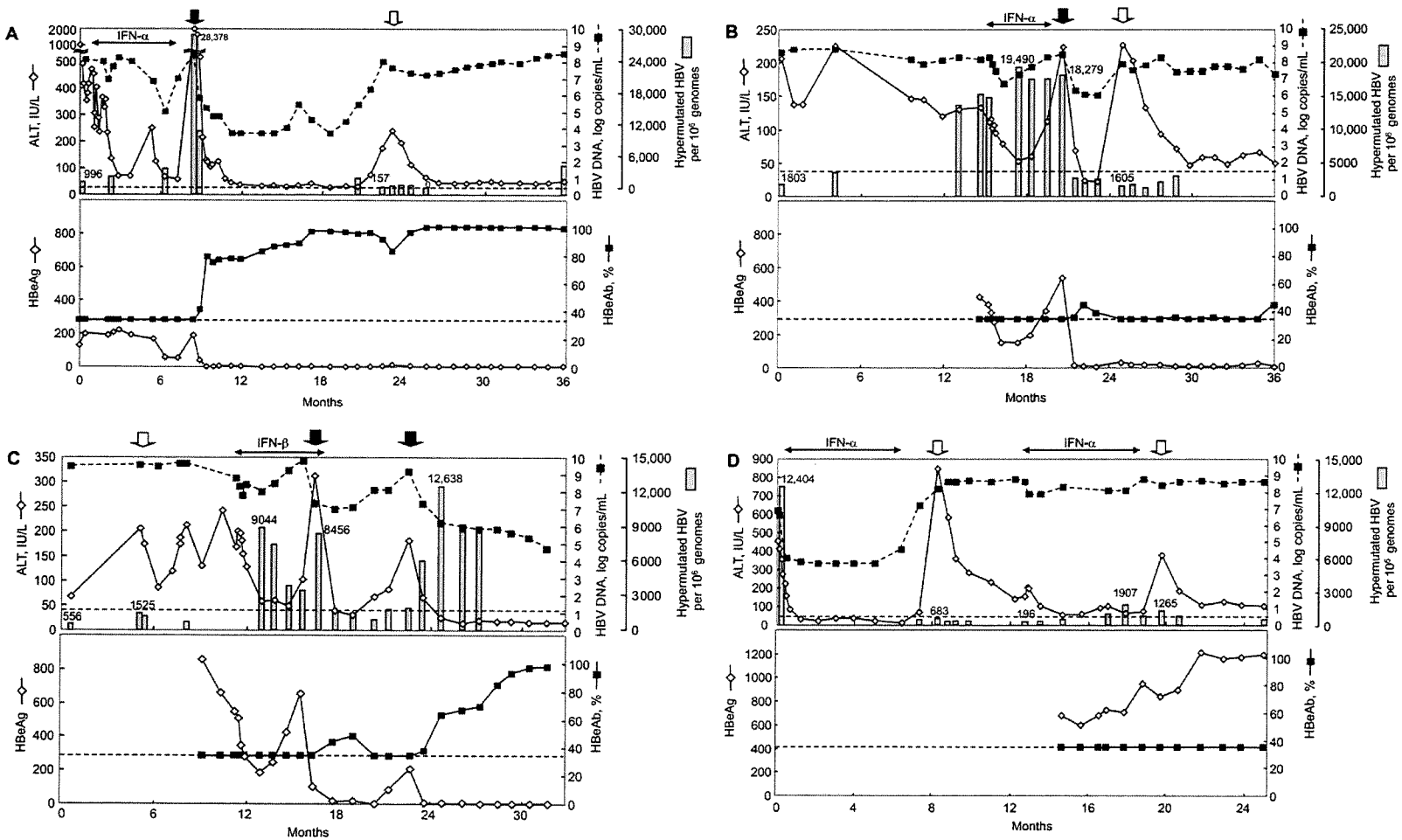

Figure 1. Clinical courses for 4 patients *(A–D)* with chronic hepatitis B virus (HBV) infection who experienced exacerbation of infection. *Black arrows,* exacerbation associated with an increase in the number of hypermutated genomes; *white arrows,* exacerbation not associated with an increase in the number of hypermutated genomes; *horizontal dotted lines,* upper normal limit of alanine aminotransferase (ALT) (40 IU/mL; *upper panel, A–D*) and the detection limit for antibody against e antigen (HBeAb) (35%; *lower panel, A–D*). HBeAb, antibody against HBV e antigen; HBeAg, HBV e antigen; IFN, interferon.

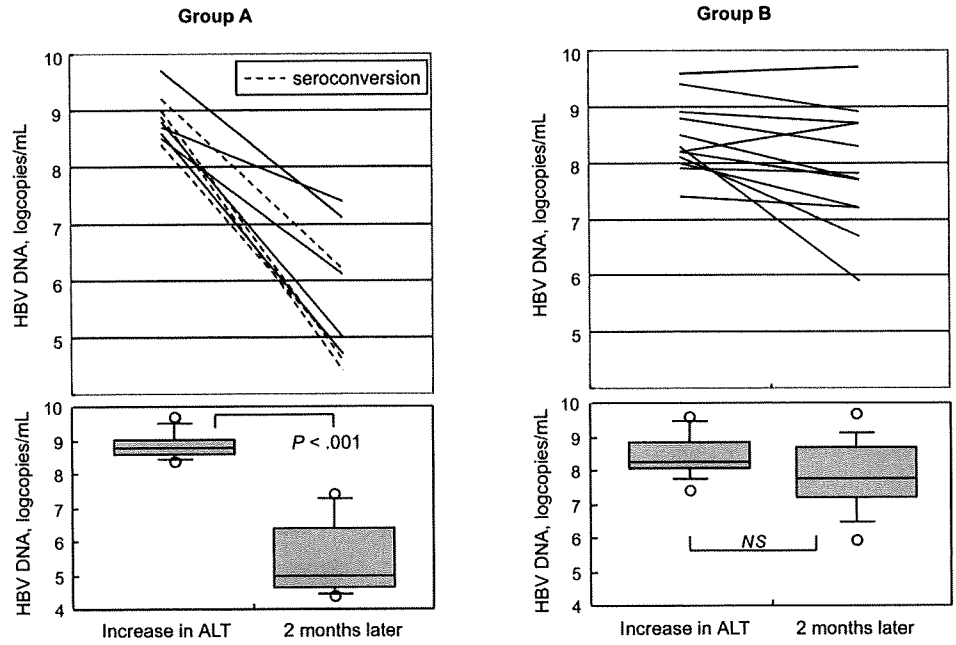

Figure 2. Relationship between increase in the number of hypermutated genomes and plasma levels of hepatitis B virus (HBV) DNA in 17 patients with chronic HBV infection who experienced >2 increases of >100 IU/L in alanine aminotransferase (ALT) level. Patients' exacerbations were divided into 2 groups, 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 12 exacerbations that involved a ≤5-fold increase in the number of hypermutated genomes). *Upper panel* for groups A and B, individual HBV DNA 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 HBV DNA levels at 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.

IFN or that the feedback system for IFN signaling was already active before initiation of therapy.

We also compared the degree of reduction in the plasma HBV DNA level for exacerbations (i.e., increases in ALT level) associated with a marked increase in the number of hypermutated genomes (i.e., those in which the peak number was >5 times the number observed prior to exacerbation) and for exacerbations not associated with such an increase. As shown in figure 2, 8 of 9 exacerbations that were coupled with a marked increase in the number of hypermutated genomes (group A) were associated with a >2-log reduction in the HBV DNA level. In contrast, only 1 of the 12 exacerbations not associated with a marked increase in the number of hypermutated genomes (group B) was associated with a >2-log reduction in plasma HBV DNA level. The median serum HBV DNA level decreased from 8.8 to 5.0 log copies/mL among the patients in group A ($P < .001$) but did not decrease for patients in group B (figure 2).

In addition, we compared the reduction in e antigen level for these 2 groups. Levels were reduced in both groups, but the median reduction was more prominent for patients in group A than for those in group B (figure 3). All 4 exacerbations coupled with e antigen seroconversion (from positive to negative) were associated with marked increase in hypermutated genomes (figure 3).

Effect of IFN treatment on the rate of HBV hypermutation in chimeric mice. Next, we examined the effect of IFN treatment on G-to-A hypermutation in HBV genomes in human hepatocyte–chimeric mice. Two mice were intravenously injected with supernatant produced by HepG2 cells transiently transfected with a plasmid containing 1.4–genome length wild-type HBV genomes. Ten weeks later, after confirmation of high-level HBV viremia, the mice were treated with 7000 IU/g/day of IFN-α, injected intramuscularly, for 14 days. We observed an ~1.5-log reduction in plasma HBV DNA level accompanied by an increase in the number of hypermutated genomes in both mice (figure 4*A*). In a mouse inoculated with HBV but treated with phosphate-buffered saline, no increase of hypermutated genomes was observed (figure 4*B*). We also observed a 36-fold increase in the level of APOBEC3G mRNA, as determined by human oligonucleotide microarray (data not shown).

Infectivity of hypermutated genomes. To study the biological significance of hypermutated genomes, culture supernatant from HepG2 cells transfected with both HBV and APOBEC3G (5 μg each) was injected into a chimeric mouse. As shown in figure 5, the culture supernatant contained a large number of hypermutated genomes. In contrast, we could not detect hypermutated genomes in the chimeric mouse inoculated with this

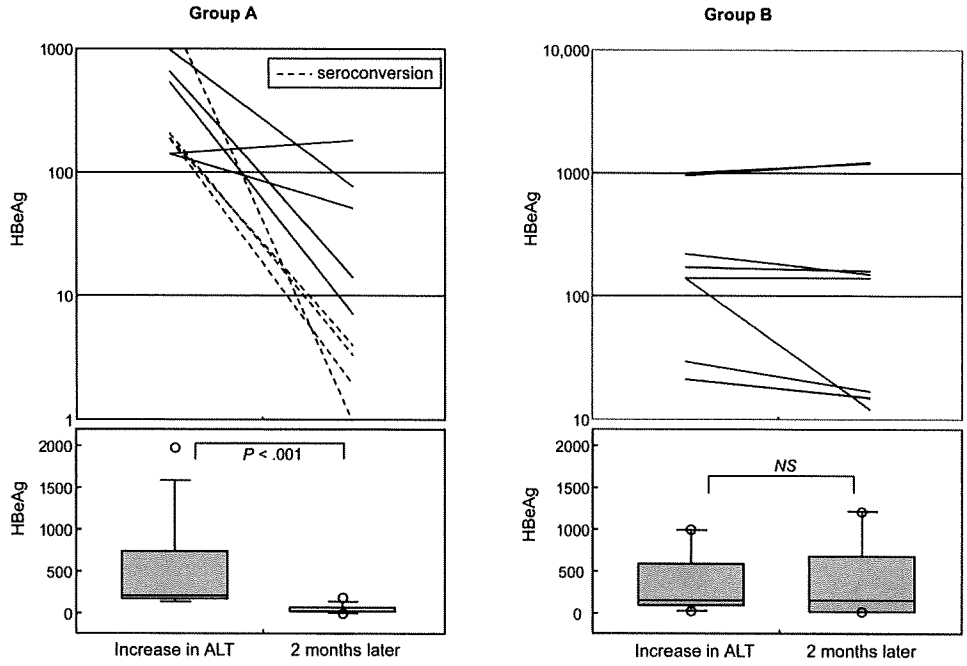

Figure 3. Relationship between increase in the number of hypermutated genomes and hepatitis B virus (HBV) e antigen (HBeAg) levels in 15 HBeAg-positive patients with chronic HBV infection who experienced >2 increases of >100 IU/L in alanine aminotransferase (ALT) level. Patients' exacerbations were divided 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 ≤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 5*A* and 5*B*). 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°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 5*C*). 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.

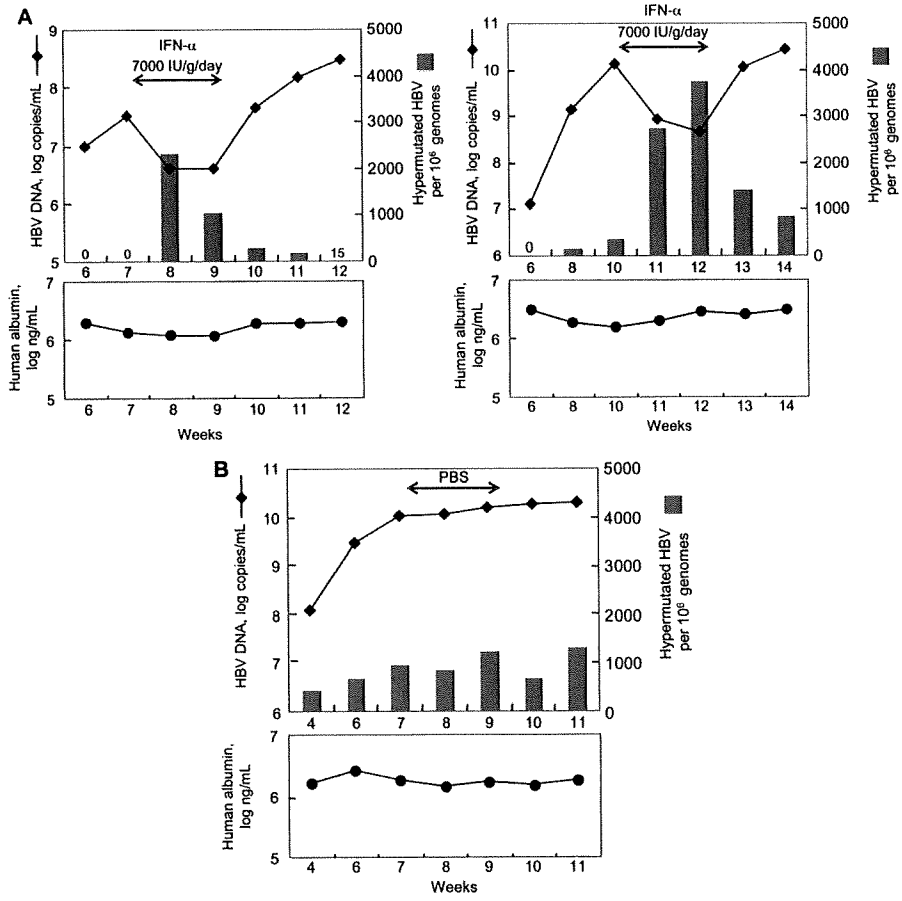

Figure 4. Effect of interferon (IFN)–α therapy on hepatitis B virus (HBV) hypermutation in HBV-infected, human hepatocyte–chimeric mice. Two chimeric mice *(A)* were inoculated with recombinant wild-type HBV produced by transfected HepG2 cells; 10 weeks later, after confirmation of high-level HBV viremia, they were treated with IFN-α 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-α 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 precore 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

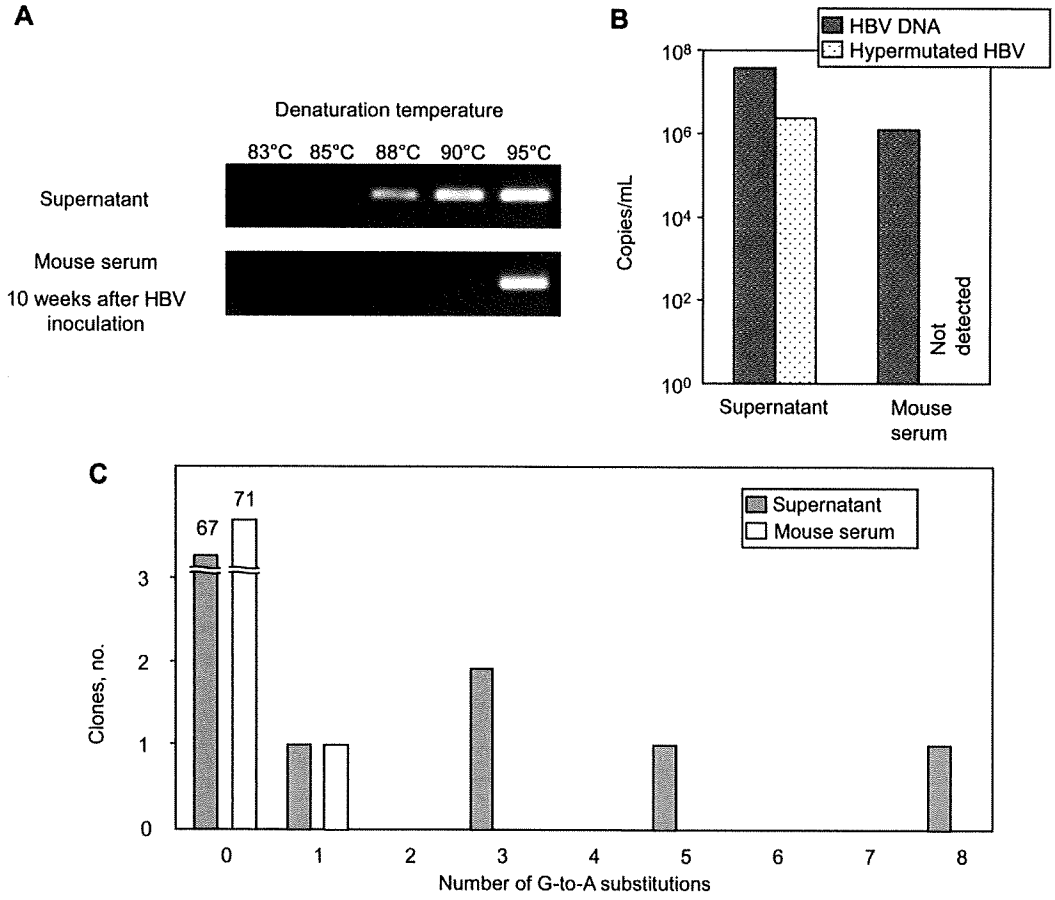

Figure 5. Results for a human hepatocyte–chimeric mouse inoculated with hepatitis B virus (HBV) produced by HepG2 cells transfected with an equal amount (5 μg each) of HBV and apolipoprotein B messenger RNA editing enzyme, catalytic polypeptide–like 3G plasmids. The inoculum contained ~6.25% hypermutated 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 10^6 HBV genomes at most (figure 1*A*). 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 genomes 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 5*C*). 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 glycocylase. 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.

Acknowledgments

We thank Rie Akiyama, Miyuki Matsushita, and Yoshiko Seo for excellent technical assistance and Yoshiko Nakata for secretarial assistance.

References

1. Wright TL, Lau JYN. Clinical aspects of hepatitis B virus infection. Lancet **1993**; 342:1340–4.
2. Ganem D, Prince AM. Hepatitis B virus infection: natural history and clinical consequences. N Engl J Med **2004**; 350:1118–29.
3. Bruix J, Llovet JM. Hepatitis B virus and hepatocellular carcinoma. J Hepatol **2003**; 39(Suppl 1):S59–63.
4. Sheehy AM, Gaddis NC, Choi JD, Malim MH. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature **2002**; 418:646–50.
5. Mangeat B, Turelli P, Caron G, Friedli M, Perrin L, Trono D. Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. Nature **2003**; 424:99–103.
6. Zhang HYB, Pomerantz RJ, Zhang C, Arunachalam SC, Gao L. The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. Nature **2003**; 424:94–8.
7. Lecossier D, Bouchonnet F, Clavel F, Hance AJ. Hypermutation of HIV-1 DNA in the absence of the Vif protein. Science **2003**; 300:1112.
8. Harris RS, Bishop KN, Sheehy AM, et al. DNA determination mediates innate immunity to retroviral infection. Cell **2003**; 113:803–9.
9. Liu B, Yu X, Luo K, Yu Y, Yu XF. Influence of primate lentiviral vif and proteasome inhibitors on human immunodeficiency virus type 1 virion packaging of APOBEC3G. J Virol **2004**; 78:2072–81.
10. Mehle A, Strack B, Ancuta P, Zhang C, McPike M, Gabuzda D. Vif overcomes the innate antiviral activity of APOBEC3G by promoting its degradation in the ubiquitin-proteasome pathway. J Biol Chem **2004**; 279:7792–8.
11. Marin M, Rose KM, Kozak SL, Kabat D. HIV-1 Vif protein binds the editing enzyme APOBEC3G and induces its degradation. Nat Med **2003**; 9:1398–403.
12. Stopak K, de Noronha C, Yonemoto W, Greene WC. HIV-1 Vif blocks the antiviral activity of APOBEC3G by impairing both its translation and intracellular stability. Mol Cell **2003**; 12:591–601.
13. Zack JA, Arrigo SJ, Weitsman SR, Go AS, Haislip A, Chen IS. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell **1990**; 61:213–22.
14. Korin YD, Zack JA. Progression to the G1b phase of the cell cycle is required for completion of human immunodeficiency virus type 1 reverse transcription in T cells. J Virol **1998**; 72:3161–8.
15. Pierson TC, Zhou Y, Kieffer TL, Ruff CT, Buck C, Siliciano RF. Molecular characterization of preintegration latency in human immunodeficiency virus type 1 infection. J Virol **2002**; 76:8518–31.
16. Stevenson M, Stanwick TL, Dempsey MP, Lamonica CA. HIV-1 replication is controlled at the level of T cell activation and proviral integration. EMBO J **1990**; 9:1551–60.
17. Chiu YL, Soros VB, Kreisberg JF, Stopak K, Yonemoto W, Greene WC. Cellular APOBEC3G restricts HIV-1 infection in resting $CD4^+$ T cells. Nature **2005**; 435:108–14.
18. Tanaka Y, Marusawa H, Seno H, et al. Antiviral protein APOBEC3G is induced by interferon-alpha stimulation in human hepatocytes. Biochem Biophys Res Commun **2006**; 341:314–9.
19. Peng G, Lei KJ, Jin W, Greenwell-Wild T, Wahl SM. Induction of APOBEC3 family proteins, a defensive maneuver underlying interferon-induced anti-HIV-1 activity. J Exp Med **2006**; 203:41–6.
20. Bonvin M, Achermann F, Greeve I, et al. Interferon-inducible expression of APOBEC3 editing enzymes in human hepatocytes and inhibition of hepatitis B virus replication. Hepatology **2006**; 43:1364–74.
21. Chen K, Huang J, Zhang C, et al. Alpha interferon potently enhances the anti-human immunodeficiency virus type 1 activity of APOBEC3G in resting primary CD4 T cells. J Virol **2006**; 80:7645–57.
22. Newman EN, Holmes RK, Craig HM, et al. Antiviral function of APOBEC3G can be dissociated from cytidine deaminase activity. Curr Biol **2005**; 15:166–70.
23. Navarro F, Bollman B, Chen H, et al. Complementary function of the two catalytic domains of APOBEC3G. Virology **2005**; 333:374–86.
24. Nguyen DH, Gummuluru S, Hu J. Deamination-independent inhibition of hepatitis B virus reverse transcription by APOBEC3G. J Virol **2007**; 81:4465–72.
25. Gunther S, Sommer G, Plikat U, Iwanska A, WainHobson S, Will H, et al. Naturally occurring hepatitis B virus genomes bearing the hallmarks of retroviral G→A hypermutation. Virology **1997**; 235:104–8.
26. Suspene R, Guetard D, Henry M, Sommer P, Wain-Hobson S, Vartanian JP. Extensive editing of both hepatitis B virus DNA strands by APOBEC3 cytidine deaminases in vitro and in vivo. Proc Natl Acad Sci U S A **2005**; 102:8321–6.
27. Noguchi C, Ishino H, Tsuge M, et al. G to A hypermutation of hepatitis B virus. Hepatology **2005**; 41:626–33.
28. Noguchi C, Hiraga N, Mori N, et al. Dual effect of APOBEC3G on hepatitis B virus. J Gen Virol **2007**; 88:432–40.
29. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology **1994**; 19:1513–20.
30. Norder H, Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of 6 strains of the hepatitis-B virus, 4 of which represent 2 new genotypes. Virology **1994**; 198:489–503.
31. Tsuge M, Hiraga N, Takaishi H, et al. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. Hepatology **2005**; 42:1046–54.
32. Tateno C, Yoshizane Y, Saito N, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am J Pathol **2004**; 165:901–12.

GASTROENTEROLOGY 2009;137:673–681

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

TAKUYA MATSUMURA,* ZONGYI HU,* TAKANOBU KATO,* MARLENE DREUX,‡ YONG-YUAN ZHANG,* MICHIO IMAMURA,§ NOBUHIKO HIRAGA,§ JEAN-MARC JUTEAU,‖ FRANCOIS-LOIC COSSET,‡ KAZUAKI CHAYAMA,§ ANDREW VAILLANT,‖ and T. JAKE LIANG*

*Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland; ‡Universite de Lyon, INSERM U758, and Ecole Normale Superieure de Lyon, Lyon, France; §Department of Medicine and Molecular Science, Division of Frontier Medical Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan; and ‖REPLICor Inc, Laval, Quebec, Canada

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.[1] 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.[2,3] In addition to other viral targets, viral entry is an attractive target for antiviral development because of the potentially conserved mechanism of viral entry.[4] Although several candidate receptors for HCV have been identified,[5–10] 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.[7,11,12] Antiviral compounds targeting the entry step of viral infection have been successfully developed in other viral infections.[13] 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.[14] 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.[14]

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_2 in Dulbecco's modified Eagle medium, containing 10% fetal bovine serum. All PS-ONs and PO-ONs were synthesized as described previously.[14] Oligonucleotides lacking the phosphorothioate modification (PO-ONs) were synthesized with the addition of 2′-O-methyl ribose modification, which stabilizes oligonucleotides from nuclease degradation.[14] 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.

0016-5085/09/$36.00
doi:10.1053/j.gastro.2009.04.048

HCV Infection and Replication Assays

The production of cell culture-generated HCV JFH-1 (HCVcc) and HCV pseudovirus (HCVpp) has been reported previously[5,15] 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.[16] 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[17] (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[18,19] 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 $CaCl_2$) buffer containing 1% bovine serum albumin as reported previously[20] 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[21] 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).[22] 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 3.9×10^3 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 ± 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[14,23] 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 1*A*) and intracellular HCV RNA quantification (Figure 1*B*). 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 1*C* 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 1*E*). 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 1*F*). 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 2*A*). Similar effects were also observed on HCV core antigen and HCV RNA levels in the culture supernatant (Figure 2*B* and *C*). In the HCVpp system, these PS-ONs also had similar inhibitory effects (Figure 2*D*).

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

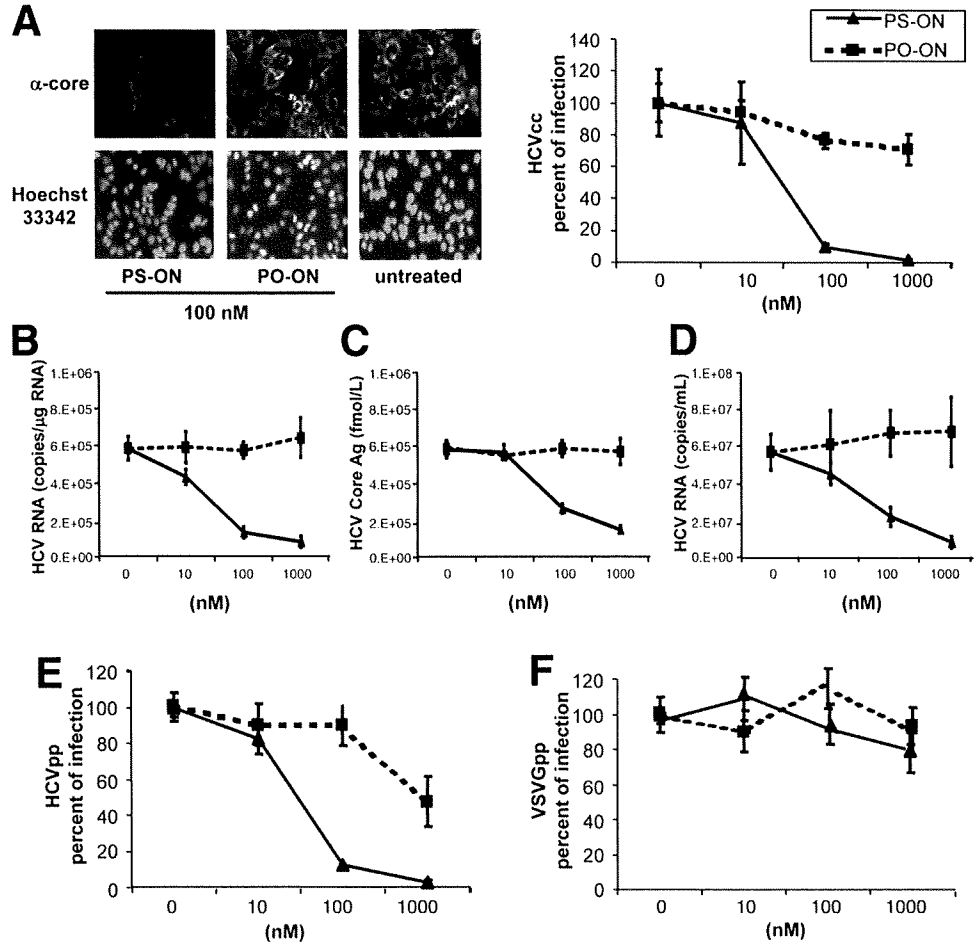

Figure 1. Effect of PS-ON on HCV infection. (*A*) Huh7.5 cells were infected with HCVcc in the presence of various concentrations of 40mer PS-ON or PO-ON (degenerate sequence). Two days after infection, infected cells were detected by immunofluorescence assay using anticore antibodies (*left panel*). 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 potently inhibited HCV infection (Figure 2*E*). 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 2*F*). 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.[16] Infections by all genotypes were equally blocked by the degenerate PS-ON, whereas the degenerate PO-ON had no effect (Figure 3*A*). Similar observation was obtained with the poly C compounds (Supplementary Figure 2*D*).

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 3*B*). 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

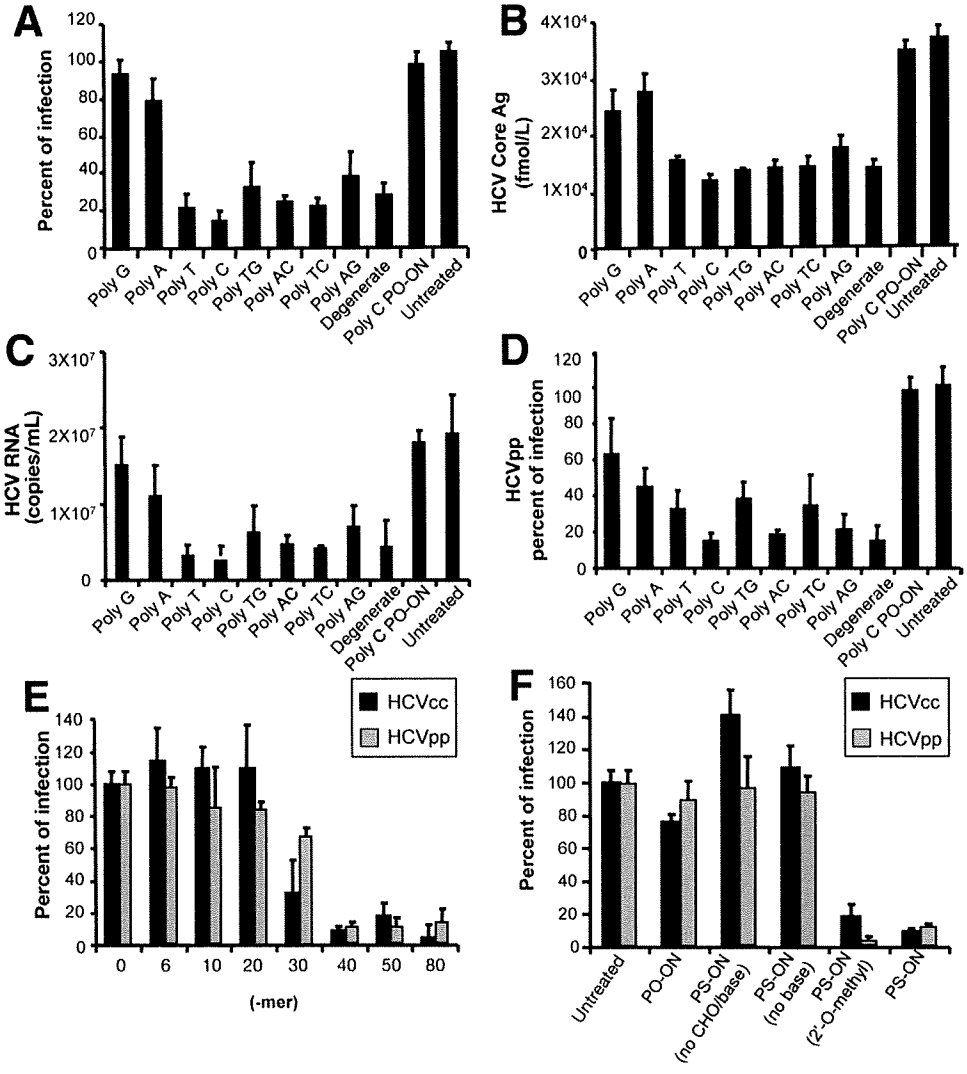

Figure 2. Sequence-independent and size- and phosphorothioation-dependent effects of PS-ON on HCV infection. A series of 40mer PS-ONs with specific sequences including poly poly G, A, T, C, TG AC, TC, and AG were tested for their inhibitory effect on HCV infection. (*A*) HCVcc was inoculated with Huh7.5 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.[24–26] 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 3*C*). 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 3*D*, 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 3*E*), 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

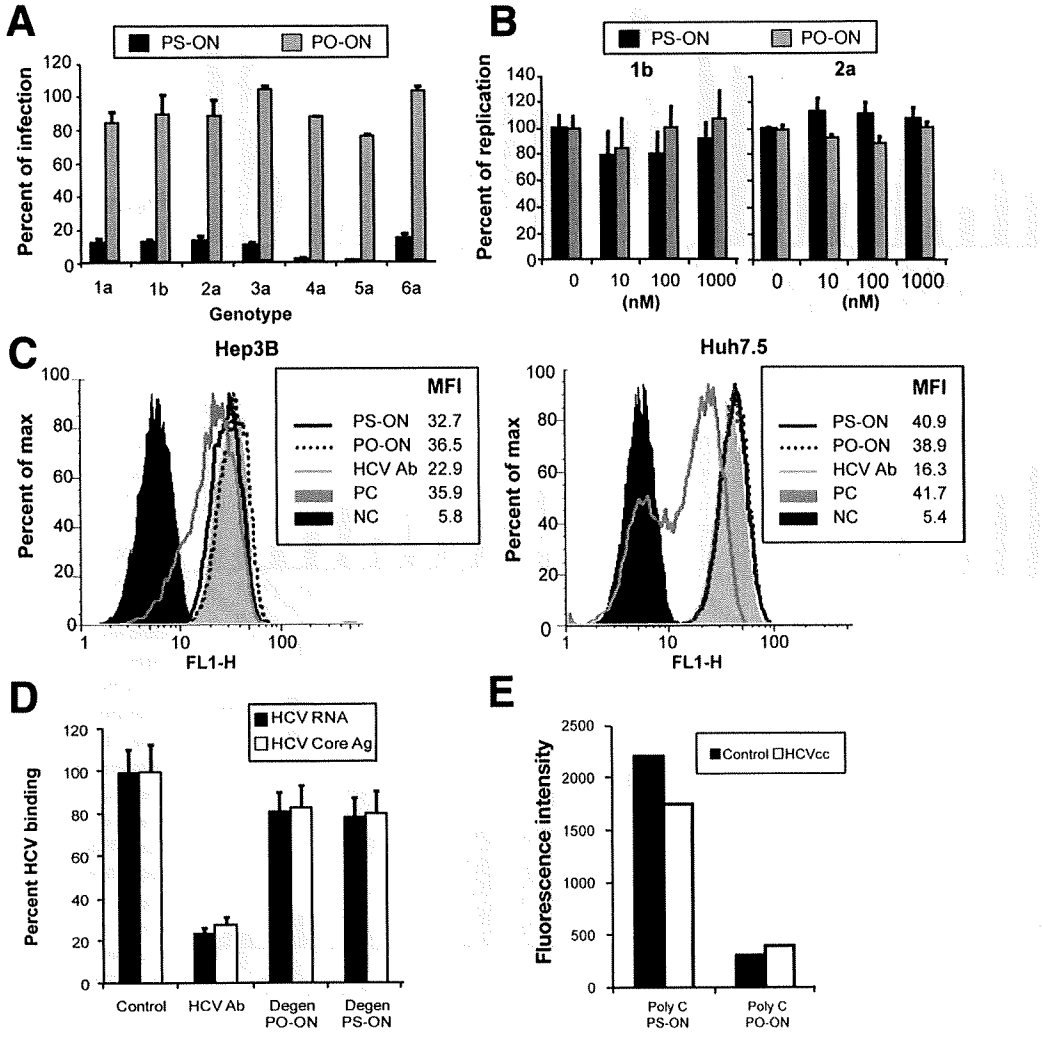

Figure 3. Effects of PS-ON on infection of various HCV genotypes, HCV replication, and cell binding. (*A*) HCVpp harboring E1/E2 glycoproteins from genotypes 1a, 1b, 2a, 3a, 4a, 5a, and 6a were inoculated into Hep3B cells and simultaneously treated with 100 nmol/L of degenerate PS-ON and PO-ON (40mer). 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 μg/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 iodixinol 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 μg/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.[12] As shown in Figure 4*A*, 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.[21] 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)[a]				PO-ON (nmol/L)[a]				AP33[b]
	0	10	100	1000	0	10	100	1000	1 μg/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

[a]Cy3 labeled degenerate PS-ON and PO-ON (40mer).
[b]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 4*B* 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,[27] 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.[28] Recent studies showed that

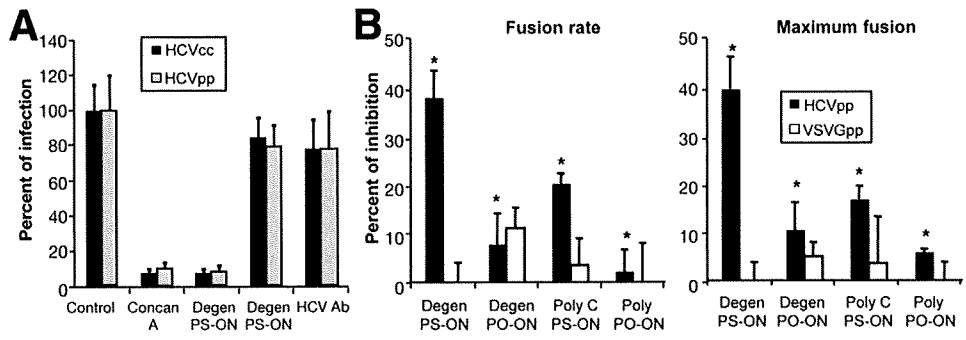

Figure 4. Effects of PS-ON on HCV viral entry. (*A*) Hep3B cells were incubated with HCVpp at 4°C for 1 hour to bind the virus and washed to remove the unbound virus. Cells were then incubated with fresh culture medium containing 25 nmol/L concanamycin A, 100 nmol/L PS-ON, 100 nmol/L PO-ON, or 25 μg/mL (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.

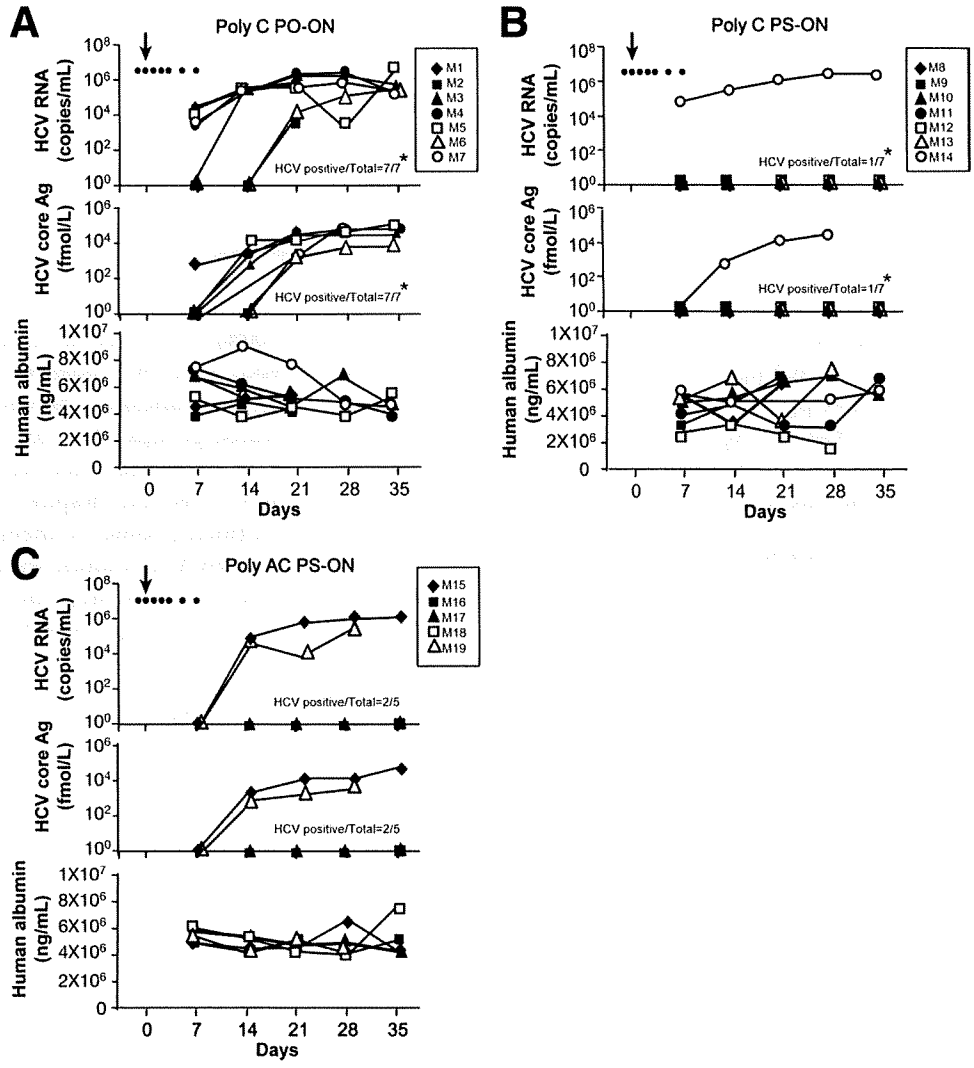

Figure 5. Effects of PS-ON on HCV infection in vivo. Human hepatocytes-transplanted uPA/SCID mice were treated intraperitoneally with 10 mg/kg of PS-ON (poly C) (n = 7) or (poly AC) (n = 5) on days −1, 0, 1, 3, 5, and 7 (indicated by *dots*). The corresponding control PO-ON (poly C) was also tested (n = 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 3.9×10^3 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,[14,29] 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.[30,31] 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.[14,23]

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.[32] 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,[33] 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.[34] 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.[34–36] It has been proposed that HCV uses a type II fusion process because of its similarity to flaviviruses.[37] Our recent study suggests that HCV and flaviviruses are indeed structurally similar.[38] 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.[39] 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.[5–10,31] CD81 and Claudin-1 have been postulated to act on the postbinding step.[6,40] 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,[41] possibly at the level of membrane fusion.[42] 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.[43] So far, prophylactic reagents based on neutralizing antibodies have been disappointing in clinical trials of liver transplantation.[44] 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, and at doi: 10.1053/j.gastro.2009.04.048.

References

1. Liang TJ, Rehermann B, Seeff LB, et al. Pathogenesis, natural history, treatment, and prevention of hepatitis C. Ann Intern Med 2000;132:296–305.
2. Feld JJ, Hoofnagle JH. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature 2005;436:967–972.
3. Pawlotsky JM. Therapy of hepatitis C: from empiricism to eradication. Hepatology 2006;43:S207–S220.
4. Smith AE, Helenius A. How viruses enter animal cells. Science 2004;304:237–242.
5. Bartosch B, Dubuisson J, Cosset FL. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. J Exp Med 2003;197:633–642.
6. Evans MJ, von Hahn T, Tscherne DM, et al. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature 2007;446:801–805.
7. Hsu M, Zhang J, Flint M, et al. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. Proc Natl Acad Sci U S A 2003;100:7271–7276.
8. Lindenbach BD, Evans MJ, Syder AJ, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623–626.
9. Lozach PY, Lortat-Jacob H, de Lacroix de Lavalette A, et al. DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. J Biol Chem 2003;278:20358–20366.
10. Scarselli E, Ansuini H, Cerino R, et al. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J 2002;21:5017–5025.
11. Bartosch B, Vitelli A, Granier C, et al. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. J Biol Chem 2003; 278:41624–41630.
12. Koutsoudakis G, Kaul A, Steinmann E, et al. Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. J Virol 2006;80:5308–5320.
13. Rusconi S, Scozzafava A, Mastrolorenzo A, et al. An update in the development of HIV entry inhibitors. Curr Top Med Chem 2007; 7:1273–1289.
14. Vaillant A, Juteau JM, Lu H, et al. Phosphorothioate oligonucleotides inhibit human immunodeficiency virus type 1 fusion by blocking gp41 core formation. Antimicrob Agents Chemother 2006;50:1393–1401.
15. Wakita T, Pietschmann T, Kato T, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791–796.
16. Lavillette D, Tarr AW, Voisset C, et al. Characterization of host-range and cell entry properties of the major genotypes and subtypes of hepatitis C virus. Hepatology 2005;41:265–274.
17. Owsianka A, Tarr AW, Juttla VS, et al. Monoclonal antibody AP33 defines a broadly neutralizing epitope on the hepatitis C virus E2 envelope glycoprotein. J Virol 2005;79:11095–11104.
18. Kato T, Date T, Miyamoto M, et al. Detection of anti-hepatitis C virus effects of interferon and ribavirin by a sensitive replicon system. J Clin Microbiol 2005;43:5679–5684.

19. Nanda SK, Herion D, Liang TJ. The SH3 binding motif of HCV (corrected) NS5A protein interacts with Bin1 and is important for apoptosis and infectivity. Gastroenterology 2006;130:794–809.
20. Triyatni M, Saunier B, Maruvada P, et al. Interaction of hepatitis C virus-like particles and cells: a model system for studying viral binding and entry. J Virol 2002;76:9335–9344.
21. Lavillette D, Bartosch B, Nourrisson D, et al. Hepatitis C virus glycoproteins mediate low pH-dependent membrane fusion with liposomes. J Biol Chem 2006;281:3909–3917.
22. Tateno C, Yoshizane Y, Saito N, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am J Pathol 2004;165:901–912.
23. Lamond AI, Sproat BS. Antisense oligonucleotides made of 2′-O-alkylRNA: their properties and applications in RNA biochemistry. FEBS Lett 1993;325:123–127.
24. Steinmann D, Barth H, Gissler B, et al. Inhibition of hepatitis C virus-like particle binding to target cells by antiviral antibodies in acute and chronic hepatitis C. J Virol 2004;78:9030–9040.
25. Baumert TF, Vergalla J, Satoi J, et al. Hepatitis C virus-like particles synthesized in insect cells as a potential vaccine candidate. Gastroenterology 1999;117:1397–1407.
26. Wellnitz S, Klumpp B, Barth H, et al. Binding of hepatitis C virus-like particles derived from infectious clone H77C to defined human cell lines. J Virol 2002;76:1181–1193.
27. Pawlotsky JM, Chevaliez S, McHutchison JG. The hepatitis C virus life cycle as a target for new antiviral therapies. Gastroenterology 2007;132:1979–1998.
28. Agrawal S, Tang JY, Brown DM. Analytical study of phosphorothioate analogues of oligodeoxynucleotides using high-performance liquid chromatography. J Chromatogr 1990;509:396–399.
29. Lee AM, Rojek JM, Gundersen A, et al. Inhibition of cellular entry of lymphocytic choriomeningitis virus by amphipathic DNA polymers. Virology 2008;372:107–117.
30. Moulard M, Lortat-Jacob H, Mondor I, et al. Selective interactions of polyanions with basic surfaces on human immunodeficiency virus type 1 gp120. J Virol 2000;74:1948–1960.
31. Barth H, Schafer C, Adah MI, et al. Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. J Biol Chem 2003;278:41003–41012.
32. Kim J, Cheong C, Moore PB. Tetramerization of an RNA oligonucleotide containing a GGGG sequence. Nature 1991;351:331–332.
33. Preiss S, Thompson A, Chen X, et al. Characterization of the innate immune signalling pathways in hepatocyte cell lines. J Viral Hepat 2008;15:888–900.
34. Kielian M, Rey FA. Virus membrane-fusion proteins: more than one way to make a hairpin. Nat Rev Microbiol 2006;4:67–76.
35. Kielian M. Class II virus membrane fusion proteins. Virology 2006;344:38–47.
36. Lescar J, Roussel A, Wien MW, et al. The fusion glycoprotein shell of Semliki Forest virus: an icosahedral assembly primed for fusogenic activation at endosomal pH. Cell 2001;105:137–148.
37. Yagnik AT, Lahm A, Meola A, et al. A model for the hepatitis C virus envelope glycoprotein E2. Proteins 2000;40:355–366.
38. Yu X, Qiao M, Atanasov I, et al. Cryo-electron microscopy and three-dimensional reconstructions of hepatitis C virus particles. Virology 2007;367:126–134.
39. Tscherne DM, Jones CT, Evans MJ, et al. Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry. J Virol 2006;80:1734–1741.
40. Cormier EG, Tsamis F, Kajumo F, et al. CD81 is an entry coreceptor for hepatitis C virus. Proc Natl Acad Sci U S A 2004;101:7270–7274.
41. Bartosch B, Verney G, Dreux M, et al. An interplay between hypervariable region 1 of the hepatitis C virus E2 glycoprotein, the scavenger receptor BI, and high-density lipoprotein promotes both enhancement of infection and protection against neutralizing antibodies. J Virol 2005;79:8217–8229.
42. Dreux M, Boson B, Ricard-Blum S, et al. The exchangeable apolipoprotein ApoC-I promotes membrane fusion of hepatitis C virus. J Biol Chem 2007;282:32357–32369.
43. Samuel D, Bizollon T, Feray C, et al. Interferon-α 2b plus ribavirin in patients with chronic hepatitis C after liver transplantation: a randomized study. Gastroenterology 2003;124:642–650.
44. Schiano TD, Charlton M, Younossi Z, et al. Monoclonal antibody HCV-AbXTL68 in patients undergoing liver transplantation for HCV: results of a phase 2 randomized study. Liver Transpl 2006;12:1381–1389.

Received December 28, 2008. Accepted April 16, 2009.

Reprint requests

Address requests for reprints to: T. Jake Liang, MD, LDB/NIDDK/NIH, Bldg 10-9B16, 10 Center Dr, Bethesda, Maryland. e-mail: JakeL@bdg10.niddk.nih.gov; fax: (301) 402-0491.

Acknowledgments

The authors thank Charles Rice, Robert Purcell, Jens Bukh, and Thomas Baumert for providing various valuable reagents.

Conflicts of interest

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

Funding

Supported in part by the Intramural Research Program of the National Institute of Diabetes and Digestive and Kidney Diseases, NIH, and a NIH Cooperative Research and Development Agreement (DK-06-0367) between NIDDK and REPLICor, Inc.

Journal of Medical Virology 81:640–649 (2009)

Randomized Trial of High-Dose Interferon-α-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

Nami Mori, Michio Imamura, Yoshiiku Kawakami, Hiromi Saneto, Tomokazu Kawaoka, Shintaro Takaki, Hiroshi Aikata, Shoichi Takahashi, Kazuaki Chayama,* and Hiroshima Liver Study Group

Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Minami-ku, Hiroshima, Japan

The aim of this study was to compare the efficacy of high-dose interferon (IFN)-α-2b with standard dose of IFN-α-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-α-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-α-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.***

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

Grant sponsor: Ministry of Health, Labor and Welfare of Japan (partially supported).

*Correspondence to: Kazuaki Chayama, MD, PhD, Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan.
E-mail: chayama@hiroshima-u.ac.jp

Accepted 11 December 2008
DOI 10.1002/jmv.21438
Published online in Wiley InterScience (www.interscience.wiley.com)