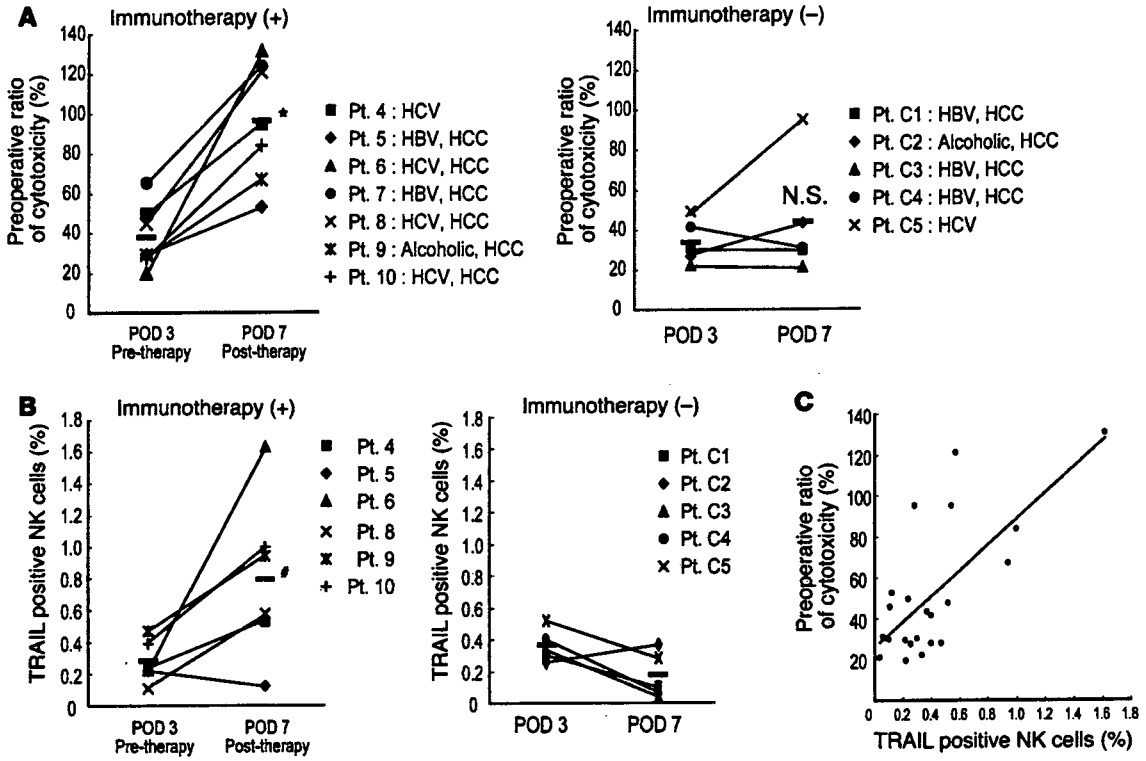

Figure 2

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

Discussion

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

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

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

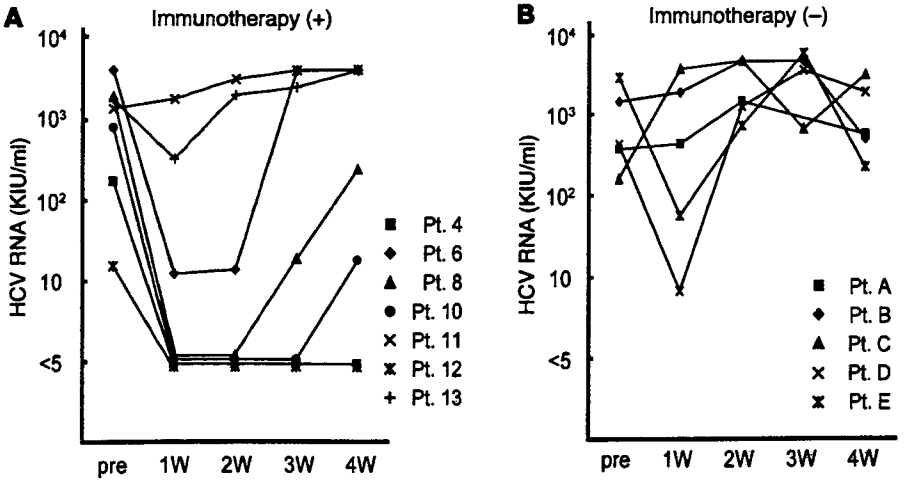

Figure 3
Serial measurement of the HCV RNA titers of LT recipients after LT. The HCV RNA titers in the sera of LT recipients who received immunotherapy were markedly lower than those in the sera of LT recipients who did not receive the therapy during the first month after LT. Each line with a different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

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

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

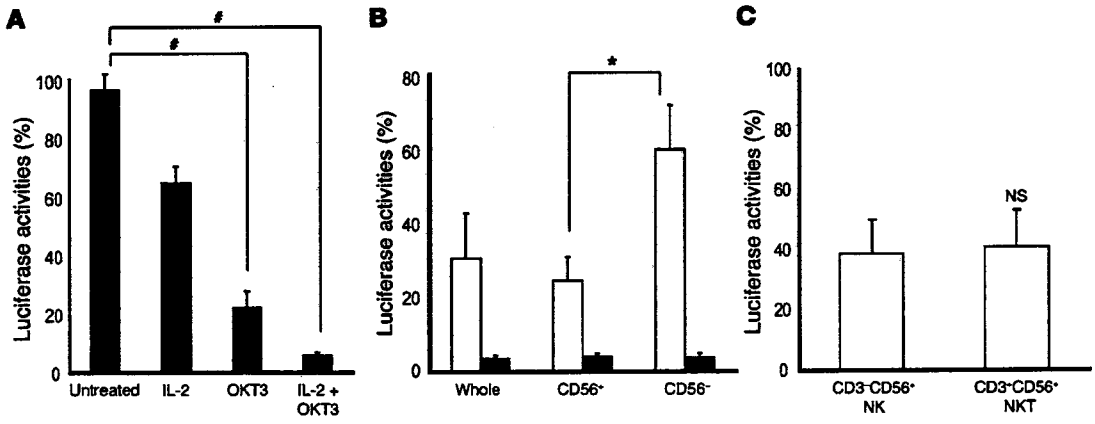

Figure 4
The cultivation of liver lymphocytes with IL-2/OKT3 markedly promoted anti-HCV activity. (**A**) Activation by IL-2 and OKT3 significantly promoted the anti-HCV effect of the liver allograft–derived lymphocytes that were cultured in complete medium with and without IL-2 (100 JRU/ml) for 3 days.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

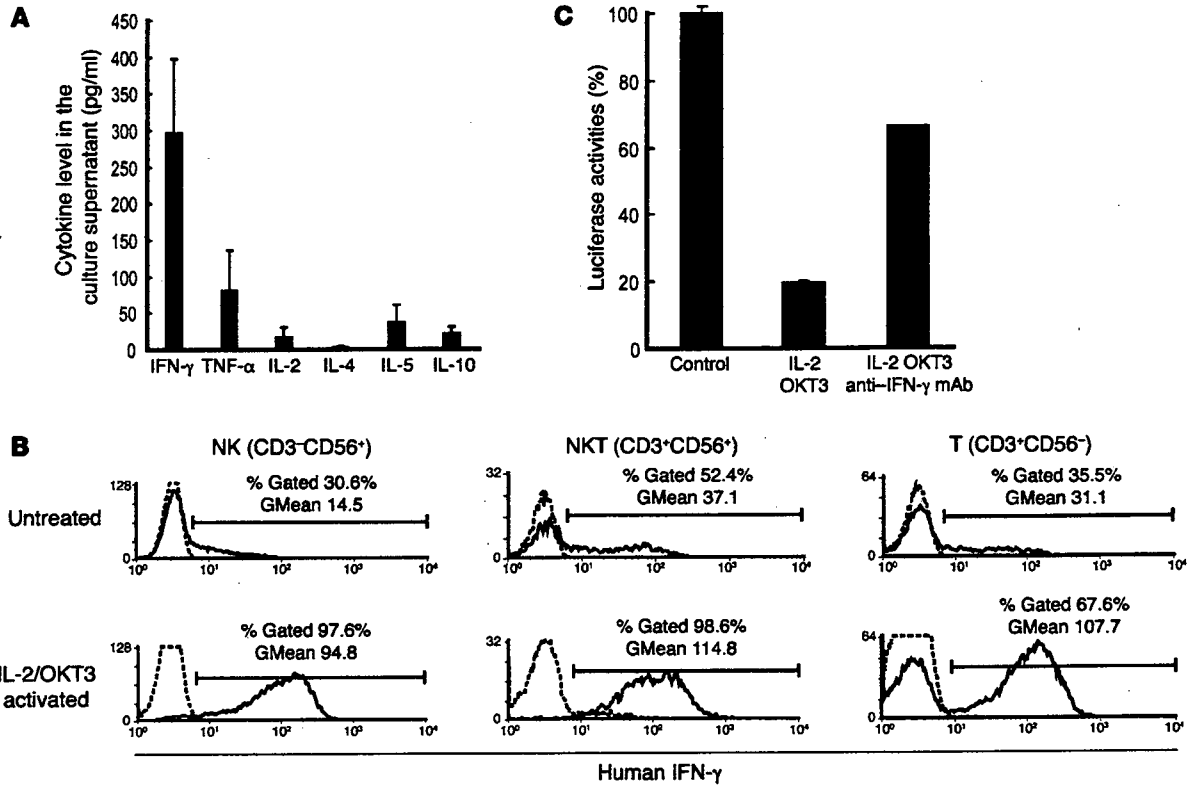

Figure 5

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

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

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

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

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

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

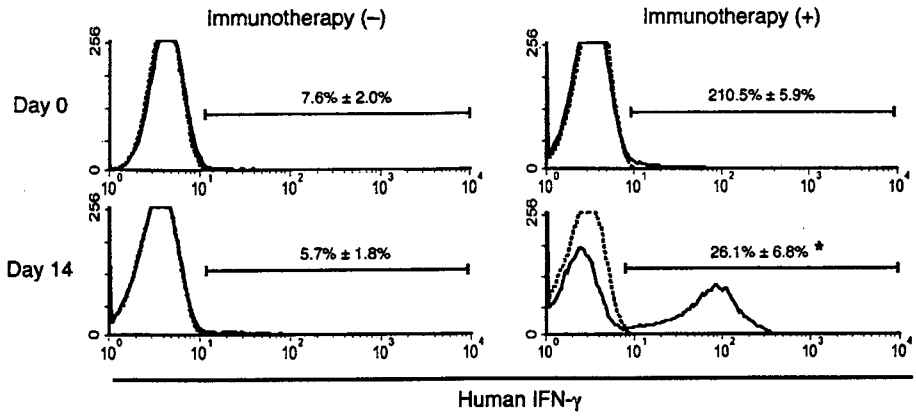

Figure 6
Adoptive immunotherapy with IL-2/OKT3–treated liver lymphocytes induced the production of IFN-γ in the LT recipients. At 14 days after LT, the number of IFN-γ–secreting cells in the peripheral blood of LT recipients treated with the adoptive immunotherapy (+) with IL-2/OKT3–treated liver lymphocytes, including NK and NKT cells, was significantly higher than that in the peripheral blood of untreated LT recipients (–). Histograms represent the proportion (percentage) of IFN-γ–positive cells among the mononuclear cells obtained from the peripheral blood of the immunotherapy (n = 4) and control group (n = 4) LT recipients. Dotted lines represent negative control staining with isotype-matched mAbs. Horizontal lines indicate the gated portion of lymphocytes. Data are presented as mean ± SEM. Histogram profiles shown are representative of 4 independent experiments. Statistical analyses were performed using the Mann-Whitney U test. *$P < 0.05$ for immunotherapy group versus control group.

Methods

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

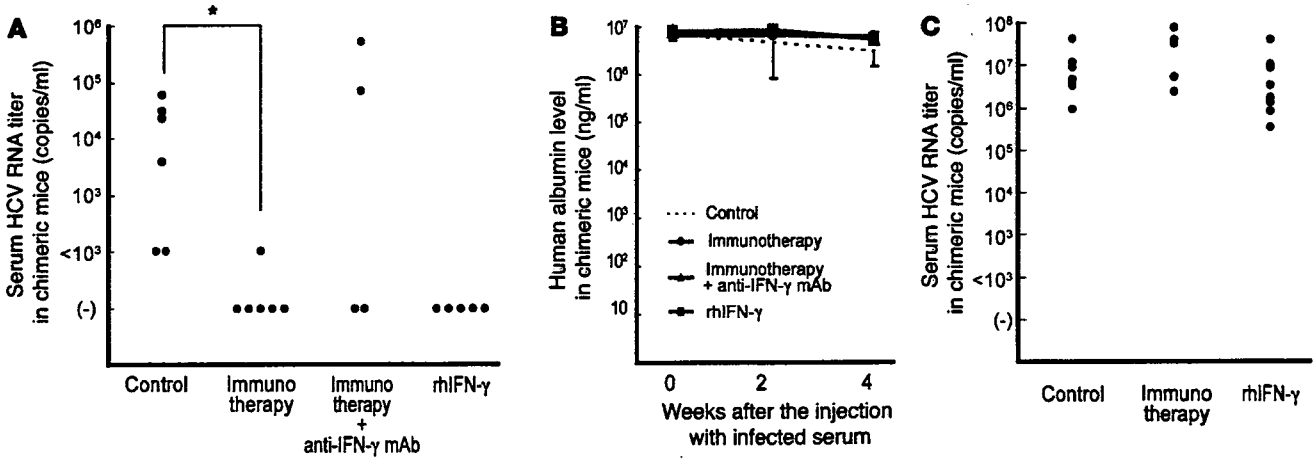

Figure 7
Adoptive immunotherapy with IL-2/OKT3–treated liver lymphocytes prevented HCV infection in human hepatocyte–chimeric mice. (**A**) Human hepatocyte–chimeric mice were intravenously injected with human serum samples positive for HCV genotype 1b. Two weeks after injecting the infected serum, the mice were intraperitoneally inoculated with IL-2/OKT3–treated liver lymphocytes (20×10^6 cells/mouse; n = 6) for adoptive immunotherapy. When indicated, anti-human IFN-γ mAb was injected intraperitoneally 1 day before the immunotherapy (n = 4). Intraperitoneal injection of recombinant human IFN-γ (rhIFN-γ) was commenced at 2 weeks after injecting the infected serum (n = 5). The untreated mice served as controls (n = 6). The dot plots represent serum HCV RNA titers in each chimeric mouse 4 weeks after the injecting the infected serum. Statistical analyses were performed using the Mann-Whitney U test. *$P < 0.01$ for immunotherapy group versus control group. (**B**) The lines represent serial changes in human serum albumin levels in the sera of the mice indicated above. Data are presented as mean ± SEM. (**C**) IL-2/OKT3–treated liver lymphocytes (20×10^6 cells/mouse) were intraperitoneally inoculated 4 weeks after the injection with the infected serum (n = 5) for adoptive immunotherapy. Intraperitoneal injection of recombinant human IFN-γ was commenced 4 weeks after the injecting the infected serum (n = 9). The untreated mice served as controls (n = 9). The dot plots represent serum HCV RNA titers in each chimeric mouse 6 weeks after injection with the infected serum.

Isolation of lymphocytes from liver allograft perfusate. Donor hepatectomy and the transplantation procedure were performed as described previously (32). After hepatectomy, ex vivo perfusion of the liver allograft was performed through the portal vein. Liver allograft–derived lymphocytes were isolated by gradient centrifugation with Ficoll-Paque (GE Healthcare Bio-Sciences AB).

Adoptive transfer of IL-2/OKT3–treated liver lymphocytes. Liver lymphocytes were cultured with human recombinant IL-2 (100 Japanese reference units/ml [JRU/ml]; Takeda) in complete medium at 37°C in a 5% CO_2 incubator for 3 days. One day before the infusion, 1 μg/ml of OKT3 (Janssen-Kyowa) was added in order to opsonize the $CD3^+$ fraction. On the day of infusion, the cells were washed twice with 0.9% sodium chloride and resuspended with 5% human serum albumin in 0.9% sodium chloride for injection (Figure 1). The viability of the cells was assessed by the dye-exclusion test, and the cells were checked twice for possible contamination by bacteria, fungi, and endotoxins.

Cytotoxicity assay. A ^{51}Cr-release assay was done as previously described (5), using HepG2 tumor cells (Japanese Cancer Research Resources Bank) as targets. Briefly, ^{51}Cr-labeled target tumor cells were added for 4 hours at 37°C to effector cells in round-bottomed 96-well microtiter plates (BD Biosciences – Discovery Labware). The percentage of specific ^{51}Cr release was calculated as follows: % cytotoxicity = [(cpm of experimental release – cpm of spontaneous release)/(cpm of maximum release – cpm of spontaneous release)] × 100. All the assays were performed in triplicate.

Flow cytometry. Flow cytometric analyses were performed using a FACSCalibur dual-laser cytometer (BD Biosciences). The following mAbs were used for the surface staining of the lymphocytes: FITC-conjugated anti-CD3 mAb (clone HIT3a; BD Biosciences – Pharmingen); PE-conjugated anti-CD56 mAb (clone B159; BD Biosciences – Pharmingen); and biotinylated anti-TRAIL (biotin-conjugated anti-TRAIL) mAb (clone RIK-2; eBioscience). The biotinylated mAb was visualized using APC-streptavidin (BD Biosciences – Pharmingen). Dead cells identified by light scatter and propidium iodide staining were excluded from the analysis. IFN-γ production in the lymphocytes was measured by a combination of cell surface and cytoplasmic mAb staining and subsequent flow cytometric analysis, as described previously (33).

Isolation of $CD56^+$ and $CD56^-$ fractions and that of NK and NKT cells. Liver allograft–derived lymphocytes were separated into a $CD56^+$ fraction – including NK and NKT cells – and a $CD56^-$ fraction by using auto MACS (Miltenyi Biotec) with anti-human CD56 microbeads (Miltenyi Biotec) according to the manufacturer's instructions. The NK and NKT cells were also isolated by magnetic cell sorting, using the human NK cell isolation kit or human $CD3^+CD56^+$ NKT cell isolation kit (Miltenyi Biotec). The purity of the isolated fractions was assessed by flow cytometric analysis, and only the fractions with purities greater than 90% were used for functional studies.

Coculture with HCV replicon–containing hepatic cells. An HCV subgenomic replicon plasmid, pRep-Feo, was derived from pRep-Neo (originally, pHCVIbneo-delS; ref. 34). The pRep-Feo carries a fusion gene comprising firefly luciferase (*Fluc*) and neomycin phosphotransferase, as described elsewhere (35, 36). After culture in the presence of G418 (Invitrogen), pRep-Feo cell lines stably expressing the replicons were established. For coculture experiments, transwell tissue culture plates (pore size, 1 μm; Costar) were used. HCV replicon-containing hepatic cells (10^5 cells) were incubated in the lower compartment with different numbers of lymphocytes in the upper compartment. The hepatic cells in the lower compartments were collected 48 hours after coculture for the luciferase assay. Luciferase activities were measured with a luminometer (Lumat LB9501; Promega), using the Bright-Glo Luciferase Assay System (Promega).

Cytometric bead array. Cytokine (IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-10) levels in the coculture assay supernatants were measured with the FACSCalibur dual-laser cytometer (BD Biosciences), using a BD Human Th1/Th2 Cytokine Cytometric Bead Array (CBA) Kit according to the manufacturer's instructions.

Generation of human hepatocyte–chimeric mice. Generation of the $uPA^{+/+}$ $SCID^{+/+}$ mice and transplantation of human hepatocytes were performed as described recently by our group (20, 37). Mouse serum concentrations of human serum albumin correlated with the repopulation index (20), and these were measured as described previously (37).

In vivo studies using human hepatocyte–chimeric mice. Human hepatocyte-chimeric mice were intravenously injected with 50 μl of the human serum samples positive for HCV genotype 1b. The serum HCV RNA titer in human hepatocyte-chimeric mice was detected by nested PCR, as previously described (38, 39). All animal protocols described in this study were performed in accordance with the guidelines and with approval of the Ethics Review Committee of Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University. Either 2 or 4 weeks after injecting the infected serum, the mice were intraperitoneally inoculated with IL-2/OKT3–treated liver lymphocytes (20×10^6 cells/mouse) for adoptive immunotherapy. When indicated, anti-human IFN-γ mAb (R&D Systems) (1.5 mg/mouse) was injected intraperitoneally 1 day before the immunotherapy. In a separate experiment, intraperitoneal injection of recombinant human IFN-γ (Imunomax-γ; Shionogi & Co. Ltd.) was commenced at either 2 or 4 weeks after injecting the infected serum. IFN-γ was administered as follows: 1×10^5 IU on the first day and thereafter 2×10^4 IU/day for 13 days.

Statistics. Data are presented as mean ± SEM. The statistical differences of the results were analyzed by 2-tailed, paired Student's *t* test, Mann-Whitney *U* test, and Mann-Whitney *U* test with Bonferroni correction after the Kruskal-Wallis *H* test, using the Stat View program. *P* values of 0.05 or less were considered statistically significant.

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Original Article

Effects of structural variations of *APOBEC3A* and *APOBEC3B* genes in chronic hepatitis B virus infection

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Aim: Human APOBEC3 deaminases induce G to A hypermutation in nascent DNA strand of hepatitis B virus (HBV) genomes and seem to operate as part of the innate antiviral immune system. We analyzed the importance of APOBEC3A (A3A) and APOBEC3B (A3B) proteins, which are potent inhibitors of adeno-associated-virus and long terminal repeat (LTR)-retrotransposons, in chronic HBV infection.

Methods: We focused on the common deletion polymorphism that spans from the 3′ part of *A3A* gene to the 3′ portion of *A3B* gene. An association study was carried out in 724 HBV carriers and 469 healthy control subjects. We also analyzed hypermutated genomes detected in deletion and insertion (non-deletion) homozygous patients to determine the effect of *APOBEC3* gene deletion. Further, we performed functional analysis of *A3A* gene by transient transfection experiments.

Results: The association study showed no significant association between deletion polymorphism and chronic HBV carrier state. Context analysis also showed a negligible effect for the deletion. Rather, mild liver fibrosis was associated with *APOBEC* gene deletion homozygosity, suggesting that *A3B* deletion is not responsible for chronic HBV infection. Functional analysis of A3A showed that overexpression of *A3A* induced hypermutation in HBV genome, although the levels of hypermutants were less than those introduced by *A3G*. However, overexpression of *A3A* did not decrease replicative intermediates of HBV.

Conclusion: These results suggest that A3A and A3B play little role in HBV elimination through anti-viral defense mechanisms. The significance of hypermutation induced by A3A should be investigated further.

Key words: APOBEC3A, APOBEC3B, APOBEC3G, deaminase, hypermutation, structural variation

INTRODUCTION

APOBEC3 CYTIDINE DEAMINASE family consists of at least seven tandem arrayed genes *APOBEC3A (A3A), A3B, A3C, A3DE, A3F, A3G,* and *A3H* on chromosome 22.[1,2] The anti-viral effect of A3G was initially identified in 2002 when it was found to inhibit the replication of human immunodeficiency virus (HIV).[3] Similarly, A3F, A3B and A3DE have been reported to inhibit HIV replication.[4–8]

APOBEC3 proteins also act on many other viruses such as simian immunodeficiency virus,[9] adeno-associated virus[10] and retrotransposons.[11–13] With regard to hepatitis B virus (HBV), A3G was also reported to inhibit HBV replication and induction of hypermutation, although the significance of the latter on viral inactivation is still controversial.[14–23] Among the APOBEC3 family members, A3B, A3C, A3G and A3F have been

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extensively analyzed in these reports for induction of hypermutation and inhibition of replication of HBV. In contrast, the function of A3A on HBV has not been evaluated despite its potent inhibitory effects on adeno-associated virus and retrotransposons.[9–13] Recently, Henry *et al.*[24] reported that, among the APOBEC3 family, A3A is the most efficient editor in induction of hypermutation in the HBV genome. This finding is not consistent with the previous reports. However, the relationship between genomic DNA editing by A3A and its effect on HBV replication have not been elucidated. This background prompted us to examine the effects of A3A on HBV replication and induction of hypermutation.

A recent study[25] identified a common deletion polymorphism of *APOBEC* gene spanning from the 3′ end of *A3A* gene to the 3′ portion of *A3B* gene (the segment extending from exon 5 of *A3A* to exon 8 of *A3B* was removed by the deletion, positions 37, 683, 131–37, 712, 716 on chromosome 22). The deletion results in complete elimination of the *A3B* coding region and the resultant fusion gene has a protein sequence identical to A3A, but has 3′ untranslated region of A3B. This polymorphism might modulate the expression levels of A3A peptide because the transcription levels and stability of this fusion mRNA could be altered by replacement of the 3′ untranslated region sequences. Analyzing the association between this deletion polymorphism and chronic HBV infection should clarify the effect of *A3B* on the establishment of chronic HBV carrier state.

The aims of the present study were to determine the association between *APOBEC3* gene deletion polymorphism and chronic HBV infection and the effect of *A3A*, which might be up- or down-regulated by the deletion polymorphism, on HBV replication and induction of hypermutation, by *in-vitro* overexpression experiments.

PATIENTS AND METHODS

Study subjects

BLOOD SAMPLES WERE obtained from 724 patients with chronic HBV infection at the hospitals of the Hiroshima Liver Study Group (http://home.hiroshima-u.ac.jp/naika1/hepatology/english/study.html) and Toranomon hospital. We also collected 469 control samples from healthy individuals who agreed to join the BioBank Japan Project at the Institute of Medical Science, the University of Tokyo. The study protocols were approved by the ethics committees of the University of Tokyo and the Center for Genomic Medicine, Riken. All participants were ethnically Japanese and provided written informed consent. Histological activity and fibrosis was assessed in liver biopsy specimens by the Metavir score.[26]

HBV markers

We measured DNA polymerase by the method of Robinson *et al.*[27] The quantity of HBV DNA was assessed by the following tests. Quantiplex HBV DNA probe assay (Chiron Corporation, Emeryville, CA), PCR (Amplicor Cobas TaqMan HBV Auto; Roche Molecular Diagnostic, Basel), transcription mediated amplification (TMA) assay (Fujirevio Diagnostic, Tokyo). The level of HBV in serum was assessed as high or low according to the following criteria (< 200 or ≥ 200 for DNA polymerase, < 200 or ≥ 200 for probe assay, < 6.0 or ≥ 6.0 for PCR assay, < 6.0 or ≥ 6.0 for TMA assay).

HBV-e antigen (HBeAg) and HBV-e antibody (HBeAb) were measured by commercially available chemiluminescent enzyme immunoassay kit (Abbott Laboratories, Chicago, IL). The cut off levels were 1.0 (cut off index) for HBeAg and 70% for HBeAb.

Genotyping

First, we genotyped genomic samples of 94 individuals by the PCR assay using the Deletion and Insertion specific primer sets reported by Kidd *et al.*[25] Since we observed some non-specific amplification, which was confirmed by sequencing analysis, we used the invader probes,[28] which specifically recognize *A3A* and *A3B*. These probes were designed and synthesized by Third Wave Technologies (Madison, WI). Deletion and two-insertion (non-deletion) PCR assays were performed separately as described previously,[25] then pooled (Deletion : Insertion1 : Insertion2 = 3:1:1), and subjected to Invader assay.

Cell culture and transfection

Human liver cancer cell line, HepG2, was purchased from RIKEN Cell Bank (Tsukuba). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C under 5% CO_2. Cells were seeded to semi-confluence in six-well tissue culture plates. Transient transfection experiments were performed using TransIT-LT1 (Mirus, Madison, WI) according to the instructions provided by the supplier.

Plasmid construction

The expression vector for hemagglutinin (HA)-tagged human A3G was kindly provided by Dr. Takaori (Kyoto University).[29] We constructed A3A cDNA expression

plasmid by cloning DNA fragment, which was amplified by PCR from cDNA obtained from lymphocytes of a deletion homozygous patient, into pcDNA3.1/nV5-DEST (Invitrogen, Carlsbad, CA). Construction of the wild-type HBV 1.4 genome length plasmid, pTRE-HB-wt was described previously (Tsuge *et al.*;[30] GenBank accession no. AB206816).

Analysis of core-associated HBV DNA

The cells were harvested 4 days after transfection and lysed with 250 l lysis buffer [10 mM Tris/HCl, pH 7.4, 140 mM NaCl and 0.5% (v/v) NP-40]. The lysate was then centrifuged for 2 min at 15 000 *g*. The core particles were immunoprecipitated from the supernatant by mouse anti-core monoclonal antibody (anti-HBc determinant , Institute of Immunology, Tokyo). Genomic DNA was separated from the core particles by SDS/proteinase K digestion followed by phenol extraction and ethanol precipitation. Quantitative analysis was performed using the above HBV DNA by RT-PCR using the RT-PCR system (Applied Biosystems, Foster City, CA). The primers and the probe used were described previously.[31] The real-time PCR was performed in a 25- l reaction volume containing 2 TaqMan Gene Expression Master Mix, 0.9 M of each primer, 0.25 M probe and 1 l DNA solution. The thermal profile was 50°C for 2 min, 95°C 10 min, followed by 40 cycles of amplification (denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec and extension at 62°C for 90 sec).

Analysis of hypermutated HBV genomes by 3D-RT-PCR

Hypermutated genomes were detected and quantified by modified 3DRT-PCR using the primers, probe and reagents described previously.[31] The thermal profile was 50°C for 2 min, 95°C for 10 min followed by initial denaturation at 85°C for 20 min and 45 cycles of amplification (denaturation at 85°C for 15 sec, annealing at 50°C for 30 sec and extension at 62°C for 90 sec).

Detection of A3A-A3B fusion mRNA by RT-PCR

We extracted total RNA from lymphocytes of each allele patients using RNeasy Mini Kit (Qiagen, Hilden) and reverse-transcribed using ReverTra Ace (TOYOBO, Osaka) with random primer in accordance with the instructions supplied by the manufacturer. We then amplified cDNAs by 35 cycles of PCR using primers specific for exon 1 of *A3A* and 3'-untranslated region of *A3B* in a 25 l reaction volume containing 1 KOD-Plus buffer [0.3 M each primers, 0.2 mM $MgSO_4$, 1 l DNA solution and 1 unit of KOD-Plus (TOYOBO Co.)]. The thermal profile was initial denaturation at 98°C for 2 min, followed by 35 cycles of amplification (denaturation at 98°C for 15 sec, annealing at 58°C for 15 sec and extension at 68°C for 60 sec). Nucleotide sequences of the amplified fusion cDNA sequences were confirmed by direct sequencing.

Western blot analysis

Cell lysates prepared as described above were separated by sodium dodecyl sulfate polyacrylamide electrophoresis on a 12% poly acrylamide gel and transferred to polyvinylidene fluoride (Pall Corporation, Pensacola, FL). The membranes were incubated with anti-V5 (Invitrogen), anti-hemagglutinin fusion epitope monoclonal anti-body (Roche) or with anti- -actin monoclonal antibody (Sigma-Aldrich, St Louis, MO) followed by incubation with horseradish peroxidase-conjugated sheep anti-mouse antibody (GE Healthcare UK, Buckinghamshire). We detected signals using the ECL system (GE Healthcare).

Nucleotide sequencing analysis of hypermutated HBV genomes by 3D-PCR, cloning and nucleotide sequencing

We analyzed hypermutated HBV DNA genomes obtained from serum samples of each genotype patient by 3D PCR (denaturation at 85°C) and cloning and sequencing. The amplified DNA fragments were cloned into pGEM T Easy vector (Promega Corporation, Madison, WI) by TA cloning. Nucleotide sequences were determined using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The nucleotide sequences were compared with those obtained by direct sequencing of amplified PCR products by normal PCR protocol.

Statistical analysis

The allele frequencies was calculated and fit to Hardy-Weinberg equilibrium was tested by the chi-square test between cases and controls using Excel software (Microsoft, Redmond, WA).[32] We also compared differences in allele frequency and genotype distribution of the deletion between cases and controls with ^{2}-test. Continuous data were compared by analysis of variance (ANOVA). Differences in categorical data were analyzed by the ^{2}-test. Differences in core-associated HBV and hypermutated HBV genomes per 1 10^4 copies of HBV genomes, were analyzed by Student's *t*-test.

Table 1 Characteristics of subjects

	Patients	Control	*P*-value
Number of patients	724	469	–
Sex			NS
Male	499	373	
Female	224	95	
Age (years)	53.1 (20.6–86.4)	55 (18–93)	NS
ALT	66 (5–3634)	–	–
Fibrosis stage		–	–
F0	13		
F1	80		
F2	149		
F3	114		
F4	46		
Activity		–	–
A0	1		
A1	50		
A2	125		
A3	47		
Platelet (10^4/mm^3)	16.5 (2.2–29.8)	–	–
HBV DNA		–	–
High	137		
Middle	108		
Low	156		
HBeAg/HBeAb		–	–
+/	207		
/+	184		
Hepatocellular carcinoma	65	–	–

Data are number of patients or median (range) values. Differences in age between case and control were compared by Mann–Whitney *U*-test. The sex ratio was analyzed by the ^{2}-test. ALT, alanine aminotransferase; HBVeAb, hepatitis B virus e antibody; HBVeAg, hepatitis B virus e antigen; NS, not significant.

RESULTS

Association between chronic HBV carriers, clinical parameters and the APOBEC3 gene deletion

TABLE 1 SUMMARIZES the clinicopathological features of the patients and control subjects. If A3B contributes to the prevention of chronic HBV infection, there should be an association between chronic HBV carrier state and *APOBEC* gene deletion polymorphism. However, we did not find any association between the two (Table 2). Furthermore, all clinical parameters, with the exception of the extent of liver fibrosis associated with chronic HBV, did not associate with the polymorphism (Tables 3,4). Advanced histopathological stages were associated with insertion homozygosity. These findings also suggest that A3B does not play any important role in anti-viral immunity in the development of chronic HBV infection.

Table 2 Case-control analysis of *APOBEC3B* deletion

	Frequency (%)		Additive mode		
	Ins	Del	*P*-value	OR	95% CI
HBV (n = 724)	0.709	0.291	0.599	0.964	0.624–1.489
Control (n = 469)	0.719	0.281			

P-values were calculated from case-control analysis by ^{2}-test. OR, odds ratio; CI, confidence interval; Del, deletion homozygote; Ins, insertion homozygote.

Table 3 Correlation between deletion and clinical parameters

	Genotype			*P*-value
	I/I	I/D	D/D	
Genotype frequency	0.50	0.42	0.08	NS
Age (years)	54.0 ± 12.8	52.0 ± 12.6	50.4 ± 13.3	NS
ALT	169.0 ± 320.6	149.5 ± 322.9	196.8 ± 309.3	NS
Platelets (10^4/mm^3)	16.8 ± 5.2	16.6 ± 6.1	17.0 ± 5.8	NS

Data are number of patients or mean ± SD. Age, ALT and platelet count were compared by ANOVA. ALT, alanine aminotransferase; D/D, deletion homozygote; H, heterozygote; I/I, insertion homozygote; NS, not significant.

Context analysis of hypermutated genomes obtained from deletion homozygous and insertion homozygous patients

The amount of hypermutated genomes was not analyzed in this study because it is known to fluctuate during the clinical course.[33] Instead, we searched for the target context of G to A mutation in hypermutated HBV genomes using serum obtained from patients with deletion homozygotes and with insertion homozygotes. As shown Figure 1, multiple G to A hypermutations were observed in deletion homozygote and insertion homozygote patients. The results of context analysis showed no significant difference between the contexts obtained from deletion homozygotes and those form non-deletion homozygotes (Fig. 2). In fact, the preferred contexts were similar in all three deletion homozygous patients and one insertion homo patient (DD1-3 and II1 in Fig. 2) but slightly different from those of the remaining two (II2 and II3). These results suggest that the effect of deletion is not strong in these preferred context patterns.

Detection of A3A-A3B fusion mRNA

We then analyzed whether the resultant *A3A* and A3B fusion was actually transcribed. We designed primers specific for exon 1 of *A3A* and the 3′-untranslated region

Table 4 Association of clinical parameters and *APOBEC* gene polymorphism (categorical data)

	Genotype frequency				Additive mode	I/I vs I/D, D/D	D/D vs I/I, I/D
	I/I	I/D	D/D				
Sex (Male/Female)				*P* value	0.76	0.85	0.30
Male (n = 328)	154 (0.47)	143 (0.44)	31 (0.09)	OR	0.75	1.03	0.72
Female (n = 166)	78 (0.47)	74 (0.45)	14 (0.08)	95% CI	0.40–1.41	0.75–1.41	0.40–1.33
Fibrosis stage (F0-F1/F2-F4)				*P* value	0.0054	0.0019	0.48
F0-F1 (n = 62)	22 (0.35)	34 (0.55)	6 (0.10)	OR	0.51	0.47	0.74
F2-F4 (n = 187)	95 (0.51)	77 (0.41)	15 (0.08)	95% CI	0.21–1.24	0.30–0.76	0.31–1.73
Activity (A0-A1/A2-A3)				*P* value	0.31	0.46	0.30
A0-A1 (n = 51)	22 (0.43)	23 (0.45)	6 (0.12)	OR	0.56	0.80	0.60
A2-A3 (n = 168)	81 (0.48)	75 (0.45)	12 (0.07)	95% CI	0.20–1.56	0.45–1.44	0.22–1.60
HBV DNA (High/Low)				*P* value	0.12	0.12	0.47
High (n = 194)	82 (0.42)	94 (0.48)	18 (0.09)	OR	0.66	0.73	0.77
Low (n = 206)	103 (0.50)	88 (0.43)	15 (0.07)	95% CI	0.32–1.40	0.49–1.09	0.38–1.57
HBeAg/HBeAb ((+/)/(/+))				*P* value	0.52	0.34	0.84
+/ (n = 207)	89 (0.43)	99 (0.48)	19 (0.09)	OR	0.96	0.82	1.07
/+ (n = 184)	88 (0.48)	78 (0.42)	18 (0.10)	95% CI	0.47–1.95	0.55–1.23	0.54–2.11
HCC				*P* value	0.85	0.89	0.64
( ) (n = 648)	323 (0.50)	266 (0.41)	59 (0.09)	OR	0.73	1.04	0.69
(+) (n = 65)	34 (0.52)	31 (0.47)	0 (0.00)	95% CI	0.25–2.13	0.62–1.73	0.24–1.98

ALT, alanine aminotransferase CI, confidence interval; D/D deletion homozygote; H, heterozygote; HBVeAg, hepatitis B virus e antibody; HBVeAg, hepatitis B virus e antigen; HCC, hepatocellular carcinoma; I/I, insertion homozygote; OR, odds ratio.

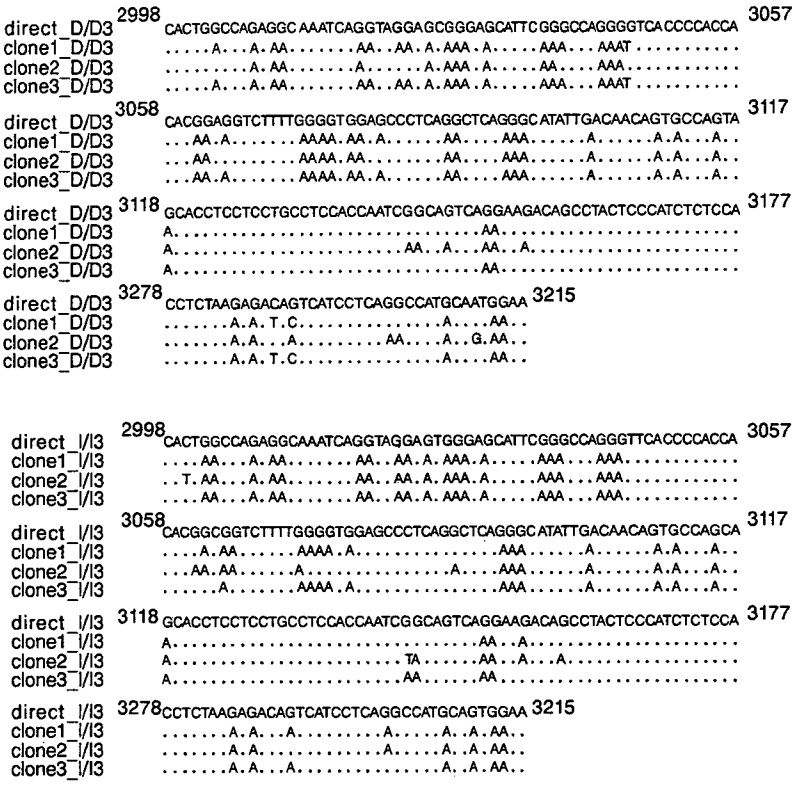

Figure 1 Nucleotide sequences of hypermutated genomes detected from deletion homozygous and insertion homozygous patients. Nucleotide sequences of 3D-PCR amplified hebatitis B virus (HBV) DNA clones are compared with those obtained by usual PCR and direct sequencing. Upper panel, nucleotide sequences obtained from a deletion homozygous patient. Lower panel, nucleotide sequences obtained from a homozygous patient. Nucleotide numbers are those from GenBank accession no. AB206816.

of *A3B*, and performed RT-PCR using cDNAs obtained from patients of each genotype. We obtained amplified DNA fragments of expected size only from deletion homozygotes and heterozygotes (Fig. 3). These results confirmed the transcription of the fusion mRNA with the coding region of *A3A* and the 3′ untranslated region of *A3B*.

Inhibition of HBV replication and induction of hypermutation by A3A

We then analyzed the antiviral effect and induction of hypermutation by A3A. Although the expression of both A3A and A3G was confirmed by western Blot analysis (Fig. 4A), transient expression of A3A did not reduce the amount of the core-associated HBV DNA in HepG2 cells (Fig. 4B). However, A3A transfection increased the hypermutated genomes of HBV in a dose-dependent manner albeit the level of induction was much lower than that observed when transfected with A3G. These results suggest that A3A has negligible anti-viral effect although it induces hypermutation of HBV genomes.

DISCUSSION

THE MAIN FINDINGS of the present study were: (i) no association between *APOBEC3* deletion and chronic HBV infection (Table 2). (ii) Mild liver fibrosis and low alanine amino transferase (ALT) levels were associated with APOBEC gene deletion homozygous genotype. (iii) The absence of *A3B* is not responsible for chronic HBV carrier status, although A3B is known as a potent inhibitor of adeno-associated virus and retrotransposons.[12] This suggests different antiviral activities for APOBEC proteins against viruses and that A3B plays little role in inhibition of HBV. (iv) The preferred context analysis showed no differences between insertion homozygotes and deletion homozygotes. Only one of the six patients examined showed different context pattern (Fig. 2). These results suggest that A3B protein has only small effect on the formation of hypermutated genomes in the serum of chronic carriers. The protein has been reported to induce hypermutation on the negative and positive strands of HBV.[18] However, our results showed that the effect of *A3B* is almost negligible in

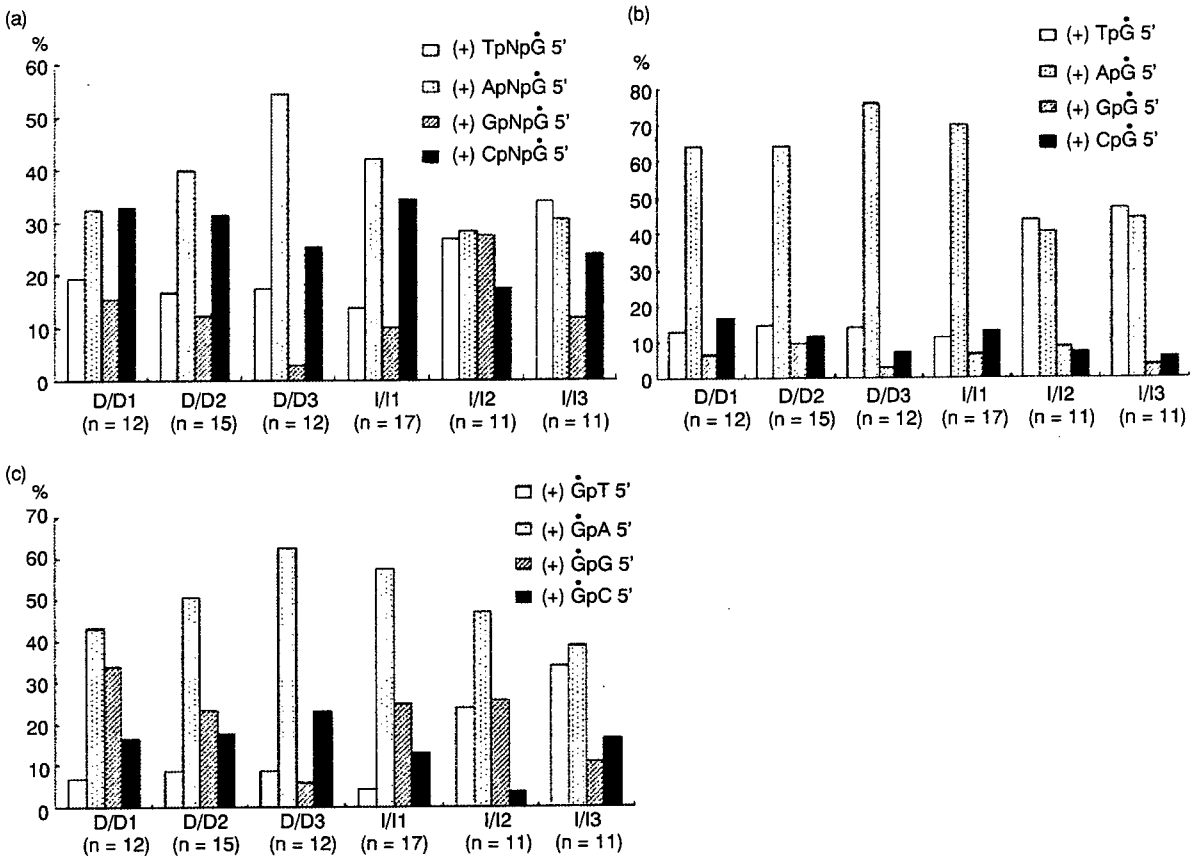

Figure 2 Context analysis of hypermutated genomes from deletion homozygous and insertion homozygous patients. Context of G to A hypermutation in hypermutated genome clones obtained from three deletion homozygous patients (D/D1, D/D2 and D/D3) and three insertion homozygous patients (I/I1, I/I2 and I/I3) were analyzed. Numbers after each patient represent the number of clones analyzed in each patient. (a) Two letters up-stream, (b) one letter upstream and (c) one letter downstream of mutated G residue were analyzed.

chronic HBV carriers compared to that of A3G. It is assumed that the other APOBEC3 family proteins mainly induce hypermutation of HBV genomes in HBV carriers to compensate for the function of deleted A3B. It is also assumed that the expression pattern of the remaining six APOBEC3 proteins is different from patient to patient.

As discussed above, our results suggest that A3B protein has almost no effect on prevention of chronic HBV infection and induction of hypermutation. It is thus assumed that A3B is not part of the innate anti-viral immune system against HBV. This is consistent with the finding that deletion is commonly seen in normal populations[25] irrespective of HBV carrier rates. Other association studies are required to clarify the role of A3B protein on other pathogens. The functional relevance of other APOBEC3 proteins on HBV infection as anti-viral immunity should be clarified further.

We also found that A3A protein induced hypermutation on the negative strand of HBV. However, the level of induction of hypermutation was much less than that of A3G (Fig. 4). Recent reports showed quite different effects for *A3A* on induction of hypermutation on HBV genomes. Henry *et al.*[24] reported that A3A is the most efficient editor of seven APOBEC3 proteins. In contrast, Zang *et al.*[23] did not detect induction of hypermutation on HBV. Although these different results might come from different cell lines and conditions used in each experiment, our results clearly showed that *A3A* induced hypermutation on the negative strand of HBV genome.

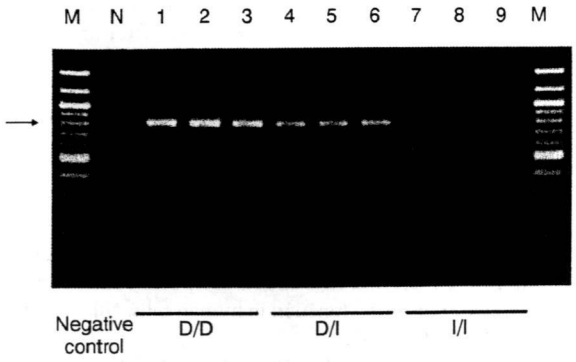

Figure 3 Detection of A3A-A3B fusion mRNA by PCR. A3A-A3B fusion mRNA was amplified by PCR using primers specific to A3A and A3B (see Materials and Methods) and detected by agarose gel electrophoresis. Lanes 1–3 are those from deletion homozygous patients, lanes 4–6 are from heterozygous patients and 7–9 are from insertion homozygous patients. M, molecular weight size marker (1 kb DNA Ladder; New England BioLabs, Ipswich, MA), N, negative control.

The deletion of APOBEC gene spans from 3′ end of the *A3A* gene to the 3′ portion of the *A3B* gene. This deletion results in the formation of a fusion gene that has *A3A* amino acid sequence and *A3B* 3′ untranslated region. The expression level of this protein may be different from the undeleted *A3A* due to different stability of RNA or different transcription levels. The results of context analysis also indicated that the effect of this alteration of expression levels of *A3A* is almost negligible compared with A3G. This is consistent with our results that showed lower levels of induction of hypermutation on HBV genome by *A3A* compared with *A3G*.

Our results indicated that the effect of both *A3A* and *A3B* is not significant in the development and progression of chronic hepatitis B. The two proteins also have only little influence on the hypermutation state of HBV in chronic HBV carriers. The results also showed higher induction levels of hypermutation by *A3G* than by *A3A*.

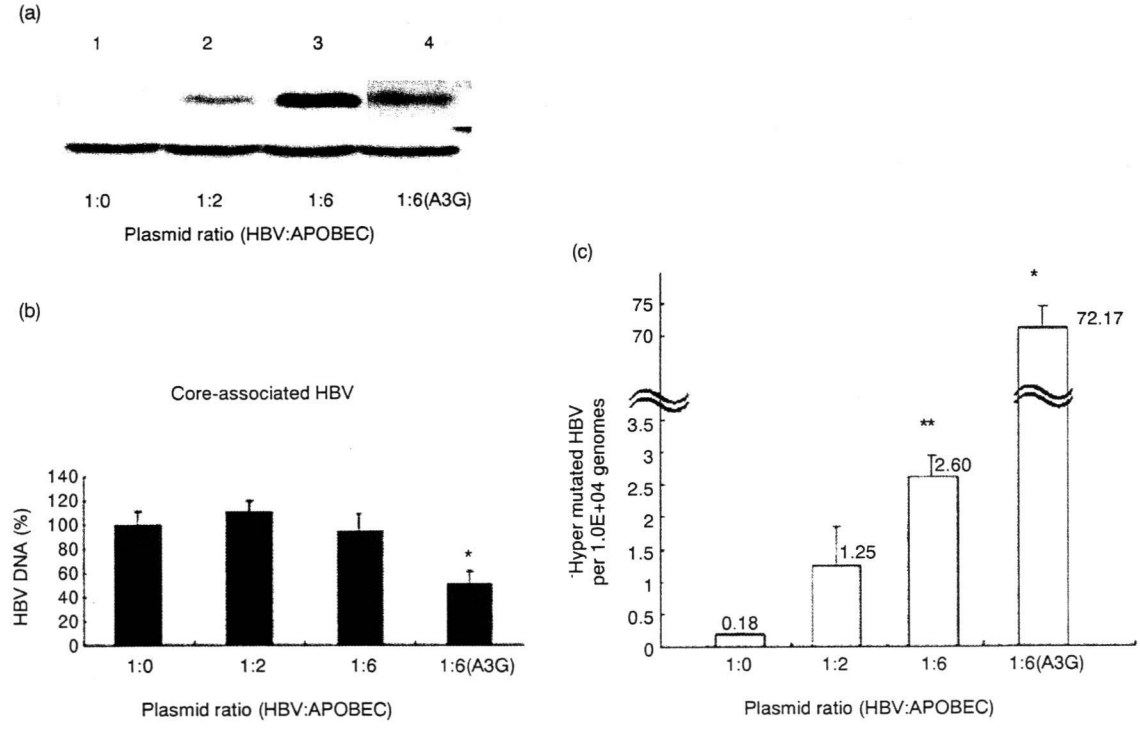

Figure 4 Analysis of inhibition of HBV replication and induction of hypermutation by A3A. HepG2 cells were transiently transfected with expression plasmid of A3A together with 1.4 genome length hepatitis B virus (HBV) expression vector. The indicated amounts of HBV and A3A (1:0, 1:2, 1:6) or A3G (1:6) expression plasmids were transfected into HepG2 cells. All experiments were performed more than twice with similar results. (a) APBEC3 gene expression levels were detected by western blot analysis. (b) The amounts of core associated replicative intermediates of HBV were measured by RT-PCR. (c) Quantitative measurement of hypermutated genomes by 3D real-time PCR. Data in (B) and (C) are mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$

Sufficient evidence indicates that *A3G* has anti-viral effects on HBV,[14–17] suggesting that some but not all APOBEC3 proteins operate as part of the anti-viral immune system against HBV infection. Further study is needed to clarify the functional role of each APOBEC3 protein for innate anti-viral immunity in chronic HBV infection.

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ORIGINAL ARTICLE

Antiviral activity, dose–response relationship, and safety of entecavir following 24-week oral dosing in nucleoside-naive Japanese adult patients with chronic hepatitis B: a randomized, double-blind, phase II clinical trial

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Abstract
Purpose A randomized, double-blind, multicenter study (ETV-047) was conducted to evaluate the dose–response relationship of entecavir and compare its antiviral activity and safety with lamivudine in Japanese patients with chronic hepatitis B (CHB).
Methods One hundred thirty-seven nucleoside-naive adult patients with CHB were randomized to once-daily oral doses of entecavir 0.01, 0.1, or 0.5 mg or lamivudine 100 mg for 24 weeks. The primary efficacy end point used to evaluate the dose–response relationship was mean change from baseline in serum hepatitis B virus (HBV) DNA level at week 22, as determined by polymerase chain reaction assay.
Results Entecavir demonstrated a clear dose–response relationship, with mean change from baseline in serum

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HBV DNA level of −3.11, −4.77, and −5.16 $\log_{10}$ copies/ml with entecavir 0.01, 0.1, and 0.5 mg, respectively. Entecavir 0.5 mg was superior to lamivudine 100 mg for the mean change in HBV DNA level (−5.16 vs. −4.29 $\log_{10}$ copies/ml; $P = 0.007$). The overall incidence of adverse events was comparable between treatment groups. Two patients discontinued treatment because of adverse events (one with liver cirrhosis [entecavir 0.5 mg] and one with grade 4 serum alanine aminotransferase (ALT) elevation, nausea, and malaise [lamivudine 100 mg]). Serum ALT flares were observed in four patients; flares were associated with 2 $\log_{10}$ reductions or more in HBV DNA level and resolved without dose interruption.

Conclusion Entecavir 0.01–0.5 mg is well tolerated and produces a dose-dependent reduction in viral load in nucleoside-naive Japanese patients with CHB. Compared with lamivudine 100 mg, entecavir 0.1 mg demonstrated noninferiority and entecavir 0.5 mg was superior in this population.

Keywords Chronic hepatitis B · Entecavir · Lamivudine · HBV DNA · ALT flare

Introduction

It is reported that more than 2 billion individuals worldwide have been infected with hepatitis B virus (HBV) and approximately 350 million people are long-term HBV carriers [1]. Chronic hepatitis B (CHB) is induced by chronic replication of HBV in the liver and has a poor prognosis, with 20–40% of infected individuals developing liver cirrhosis, noncompensated liver disorder, or hepatocellular carcinoma [2]. Treatment of CHB is aimed at sustained inhibition of HBV replication and remission of liver disease [3], ultimately preventing progression to liver cirrhosis or hepatocellular carcinoma [4].

Prior to the advent of the nucleoside analog lamivudine, interferon-α formed the mainstay of treatment, but this immunoregulatory cytokine requires parenteral administration and is poorly tolerated [5]. Lamivudine is well tolerated on oral administration and has been proven to be highly effective in the treatment of CHB, but the emergence of resistance mutations (including the YMDD motif) in the reverse-transcriptase domain of HBV polymerase frequently results in overt viral rebound and disease progression [6–9]. The novel nucleoside analog adefovir is effective against wild-type HBV and lamivudine-resistant strains and is well tolerated on long-term administration, but its clinical use is restricted by the need for renal monitoring in patients with impaired renal function [10].

Entecavir, a cyclopentylguanine-derived nucleoside analog and selective inhibitor of HBV replication, was approved by the U.S. Food and Drug Administration in 2005 for the treatment of CHB. Entecavir displays potent antiviral activity in the woodchuck and duck models of HBV infection [11, 12] and is reported to be 100- to 2,200-fold more potent than lamivudine and adefovir in inhibiting HBV replication in vitro [13, 14]. Phase II clinical trials of entecavir conducted in non-Japanese patients with CHB have demonstrated entecavir to be well tolerated and more effective than lamivudine [15, 16].

A global dose-finding study (ETV-005) conducted in lamivudine-naive patients with CHB compared three doses of entecavir (0.01, 0.1, and 0.5 mg once daily) with lamivudine 100 mg once daily over a 22-week treatment period. Entecavir showed a clear dose–response relationship and was well tolerated at all three dose levels; in addition, 0.1 and 0.5 mg of entecavir showed superior antiviral activity compared with 100 mg of lamivudine [15].

Phase I studies of single-dose (0.05–2.5 mg) and multiple-dose (0.1–1.0 mg daily) entecavir conducted in Japan have confirmed the drug's safety in healthy men. As in Caucasian populations, entecavir displayed linear plasma pharmacokinetics over a wide range of doses, including putative therapeutic doses (0.5 and 1.0 mg), in Japanese subjects; there was no evidence of significant ethnic differences in its pharmacokinetics and pharmacodynamics. Similar findings to those obtained in the global phase II clinical trials of entecavir might therefore be expected from corresponding studies conducted in Japanese patients.

To evaluate the dose–response relationship, the antiviral activity and safety of entecavir in Japanese CHB patients, we conducted a 24-week phase II study comparing entecavir (0.01, 0.1, and 0.5 mg daily) to lamivudine (100 mg daily).

Materials and methods

Study design

This randomized, double-blind, double-dummy study was conducted at 38 institutions in Japan from August 2003 to March 2005. Eligible patients comprised 20- to 75-year-old men and women with CHB who fulfilled the following criteria: (i) HBsAg-positive for 24 weeks or more or IgM HBcAb-negative with biopsy-confirmed CHB; (ii) HBeAg-positive or HBeAg-negative for 12 weeks or more; (iii) serum HBV DNA level 40 MEq/ml or more (143 pg/ml) by Quantiplex™ branched DNA hybridization method (bDNA assay) (≥7.6 $\log_{10}$ genome equivalent by the transcription-mediated amplification method or $\geq 10^{7.6}$ copies/ml by Roche Amplicor™ polymerase chain reaction method [PCR assay]) measured 2 weeks or more before screening and serum HBV DNA level 40 MEq/ml or more (by bDNA assay) at screening; (iv) serum alanine

aminotransferase (ALT) level 1.25–10 times the upper limit of normal (ULN); and (v) well-compensated liver disease with prothrombin time prolongation 3 s or less or international normalized ratio 1.5 or less, serum albumin level 3.0 g/dl or more, and total bilirubin 2.5 mg/dl or less (42.75 μmol/l). After a 6-week screening period, eligible patients were stratified according to HBeAg status and study site and randomized (1:1:1:1) to oral treatment with entecavir (0.01, 0.1, or 0.5 mg plus matching placebo capsule) or lamivudine (100 mg plus matching placebo tablet) once daily for 24 weeks. All doses were administered at fixed times of the day, avoiding the 2 h before and after meals. Pregnant women were excluded from the study, as were patients with liver cirrhosis, patients with a history or evidence of variceal bleeding, patients with hepatic encephalopathy or ascites requiring diuretics, or patients with paracentesis. Patients with other liver disease (e.g., autoimmune hepatitis) were excluded from the study. In addition, patients were excluded if they had a serum creatinine level more than 1.5 × ULN, hemoglobin level less than 10.0 g/dl, platelet count less than 70,000/mm^3, granulocyte count less than <1,500/mm^3 or plasma α-fetoprotein level more than 100 ng/ml, a history of allergy induced by nucleoside analog or exposure to nucleoside analogs, a recent history (previous 24 weeks) of treatment with immunosuppressives or interferon-α/β, or current treatment of CHB.

Treatment efficacy was assessed after 22 weeks, and all eligible patients who completed 24 weeks of blinded therapy were given the option of enrolling in a separate entecavir trial. Patients who discontinued therapy prematurely were followed up for 24 weeks postdosing. Patients began anti-HBV therapy as recommended by their physician during the postdosing follow-up period.

Informed consent was obtained from all patients in writing prior to their inclusion in the study. The study was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice guidelines and notifications were issued by the Ministry of Health and Labor.

Efficacy and safety assessment

The primary efficacy end point for the evaluation of the dose–response relationship of entecavir was the change from baseline in mean serum HBV DNA level at week 22, as determined by PCR assay. Secondary efficacy end points for the assessment of the noninferiority of entecavir at each dose to lamivudine included the change from baseline in mean serum HBV DNA level at week 22, as determined by PCR assay, the percentage of patients with a reduction in serum HBV DNA level 2 $\log_{10}$ copies/ml or more or a serum HBV DNA level below the limit of detection (400 copies/ml by PCR assay; 2.5 pg/ml or 0.7 MEq/ml by bDNA assay) at week 22, the percentage of patients with HBeAg loss, the percentage of patients with HBeAg seroconversion (HBeAg loss and appearance of HBe-antibody), the percentage of patients achieving ALT normalization (World Health Organization grade 0: <1.25 × ULN), and the percentage of patients achieving a protocol-defined response (HBV DNA level <0.7 MEq/ml by bDNA assay, HBeAg negativity and serum ALT level <1.25 × ULN for HBeAg-positive patients; HBV DNA level <0.7 MEq/ml by bDNA assay and serum ALT level <1.25 ULN for HBeAg-negative patients) at week 22. The incidence of genotypic drug resistance was also assessed in patients who had a 1 $\log_{10}$ copies/ml or more increase in HBV DNA by PCR from nadir while on study drug.

Based on the results of the global dose–response study of entecavir conducted in nucleoside-naive patients (ETV-005 study) [15], noninferiority of entecavir 0.1 or 0.5 mg compared with lamivudine (100 mg) was confirmed if the upper 95% confidence interval (CI) for the difference in mean HBV DNA levels at week 22 was 0.8 $\log_{10}$ copies/ml or less.

Assay methods

Serum HBV DNA level was determined by Roche Amplicor™ PCR assay (Roche Diagnostics K.K., Tokyo, Japan) and Quantiplex™ (Chiron) bDNA assay. Clinical laboratory tests, serum HBV DNA assays, and HBV serology were performed at the central clinical laboratory designated by the trial sponsor. Genotypic analysis of HBV isolates was performed using samples collected from patients on the first day of treatment. Genotypic analysis of HBV DNA polymerase was performed at SRL Inc. (Tokyo, Japan).

Statistical analysis

Numerical data were expressed by descriptive statistics. Serum HBV DNA level, a continuous variable, was analyzed after logarithmic transformation. For treatment group, comparisons of continuous variables, analysis of variance models, incorporating baseline HBV DNA level and HBeAg status as covariates were employed. For intertreatment comparisons of binary data, Cochran–Mantel–Haenszel tests were employed using baseline HBeAg status as a stratification factor. For analysis of dose–response relationships, Student's t test was applied to linear regression plots of serum HBV DNA level against log dose. A two-sided $P < 0.05$ was taken to indicate statistical significance. For analysis of dose–response relationships using efficacy data, a two-sided $P < 0.05/3$ was taken to

indicate statistical significance following Bonferroni adjustment.

Results

Study population and demographic characteristics

A total of 137 patients, including 20- to 73-year-old men and women, met the study eligibility criteria and were randomized to the following treatment groups: entecavir 0.01 mg ($n = 35$), entecavir 0.1 mg ($n = 34$), entecavir 0.5 mg ($n = 34$), and lamivudine 100 mg ($n = 34$). Three patients (two in the entecavir 0.5 mg group and one in the lamivudine 100 mg group) discontinued the study prematurely; the reasons for discontinuation were noncompliance (one patient in the entecavir 0.5 mg group) and adverse events (liver cirrhosis in one patient [entecavir 0.5 mg group] and grade 4 serum ALT elevation with nausea and malaise in one patient [lamivudine 100 mg group]). Accordingly, a total of 134 patients (entecavir 0.01 mg group, 35 patients; entecavir 0.1 mg group, 34 patients; entecavir 0.5 mg group, 32 patients; and lamivudine 100 mg group, 33 patients) completed 24 weeks of treatment and were included in the efficacy assessment.

The four treatment groups were matched with respect to gender, age, body weight, and proportion of HBeAg-positive patients (Table 1). Serum HBV DNA levels by PCR assay (mean ± SD) at baseline were 7.94 ± 0.87, 8.09 ± 1.05, 8.39 ± 0.73, and 7.94 ± 0.83 $\log_{10}$ copies/ml for the entecavir 0.01, 0.1, and 0.5 mg and lamivudine 100 mg groups, respectively. With regard to HBV genotype, 124 patients were genotype C, 6 patients were genotype A, 5 patients were genotype B, and 2 patients were genotype F. All patients were nucleos(t)ide-naive and none had been pretreated with interferon therapy.

Virologic response

Mean changes (from baseline) in serum HBV DNA level at week 22 were −3.11, −4.77, and −5.16 $\log_{10}$ copies/ml with entecavir 0.01, 0.1, and 0.5 mg, respectively (Fig 1; Table 2). Estimated differences in serum HBV DNA levels between the 0.1 and 0.5 mg entecavir groups and the low-dose entecavir group (0.01 mg) were determined after adjustment for baseline level and HBeAg status. Estimated intertreatment group differences (adjusted 95% CI) were −1.61 (−2.20 to −1.02) $\log_{10}$ copies/ml between the entecavir 0.01 and 0.1 mg groups and −1.95 (−2.53 to −1.37) $\log_{10}$ copies/ml between the entecavir 0.5 and 0.01 mg groups; both of these differences were statistically significant ($P < 0.0001$). In contrast, the difference in serum HBV DNA levels between the high-dose (0.5 mg) and medium-dose (0.1 mg) entecavir groups was not statistically significant (estimated difference [adjusted 95% CI] −0.23 [−0.69 to 0.23] $\log_{10}$ copies/ml). Taken together, these results demonstrate the superiority of high- and medium-dose entecavir (0.1 and 0.5 mg) compared with low-dose entecavir (0.01 mg) in terms of viral load reduction (Table 3). Linear regression analyses indicated a

Table 1 Baseline demographics and clinical characteristics of treated subjects

	ETV 0.01 mg ($n = 35$)	ETV 0.1 mg ($n = 34$)	ETV 0.5 mg ($n = 34$)	LVD 100 mg ($n = 34$)
Male, *n* (%)	25 (71.4)	23 (67.6)	23 (67.6)	28 (82.4)
Female, *n* (%)	10 (28.6)	11 (32.4)	11 (32.4)	6 (17.6)
Age (years), mean ± SD	42.0 ± 12.5	40.1 ± 9.8	39.8 ± 10.4	42.3 ± 12.6
Weight (kg), mean ± SD	66.2 ± 12.5	64.6 ± 11.9	65.3 ± 11.1	64.4 ± 9.0
Ethnicity Japanese, *n* (%)	35 (100)	34 (100)	34 (100)	34 (100)
HBV DNA ($\log_{10}$ copies/ml by PCR), mean ± SD	7.94 ± 0.87	8.09 ± 1.05	8.39 ± 0.73	7.94 ± 0.83
HBeAg positive, *n* (%)	30 (85.7)	30 (88.2)	30 (88.2)	31 (91.2)
ALT (IU/l), mean ± SD	150.1 ± 111.8	162.0 ± 127.1	142.4 ± 82.2	185.0 ± 130.8
AST (IU/l), mean ± SD	83.2 ± 40.0	114.3 ± 109.4	81.0 ± 43.0	121.6 ± 85.4
Total bilirubin (mg/dl), mean ± SD	0.65 ± 0.25	0.56 ± 0.15	0.66 ± 0.25	0.71 ± 0.28
HBV genotype (%)				
C	32 (91.4)	30 (88.2)	32 (94.1)	30 (88.2)
A	1 (2.86)	2 (5.88)	1 (2.94)	2 (5.88)
B	1 (2.86)	1 (2.94)	1 (2.94)	2 (5.88)
F	1 (2.86)	1 (2.94)	0	0

ETV entecavir; *LVD* lamivudine