Table 1 Data of the using primer sets

Primer name	Sequence	Expected size of amplification
HCV-P1	Sense: 5′-CGCCGACCTCATGGGGTACA-3′ Antisense: 5′-TGGTGTACATTTGGGTGATT-3′	2919 bp
HCV-P2	Sense: 5′-TGCTCTTTCTCTATCTTCCT-3′ Antisense: 5′-GTGATGATGCAACCAAGTAG-3′	2618 bp
HCV-P3	Sense: 5′-TCTTTGCCGGCGTTGACGGG-3′ Antisense: 5′-TAGGGTGGTGAAGGAACAGG-3′	957 bp

Table 2 Baseline characteristics of patients ($n = 23$)

Age (years)*	54.5 (39–68)
Gender (male/female)	12/11
Disease for LDLT (LC/LC + HCC)	12/11
Fibrosis grade before therapy (F0/F1/F2/F3)	7/10/5/1
Alanine aminotransferase (IU/L)*	191.9 (53–845)
White blood cell counts (per mm^3)*	4500 (1300–9600)
Haemoglobin (g/dL)*	12.2 (9.80–14.7)
Platelet counts ($\times 10^4/mm^3$)*	17.1 (5.10–47.7)
Prothrombin time (INR)*	1.20 (0.87–3.09)
Total bilirubin (mg/dL)*	2.43 (0.50–13.4)
Creatinine (mg/dL)*	0.77 (0.50–1.60)
Virus genotype (1b/2a/2b/others)	20/1/1/1
Pretreatment viral loads (kIU/mL)	
<100	0
100–850	6 (120, 347, 357, 630, 800, 830)†
>850	17 (860, 880, 920, 950, 1020, 1060, 1420, 1470, 1790, 1920, 1940, 1960, 2320, 2940, 3210, 4730, 21200)†
IFN dosage (300 MU/600 MU)	9/14

LDLT, living-donor liver transplantation; LC, liver cirrhosis; HCC, hepatocellular carcinoma; IFN, interferon; MU, million units.
*Values are median (range).
†Actual viral load of each patient.

Statistical analysis

Results are expressed as mean or median and range (minimum and maximum). Pretreatment values were compared using the Mann–Whitney *U*-test. Categorical variables were analysed by Fisher's exact test. $P < 0.05$ was considered statistically significant. *P* values of HLA data were corrected by multiplying the number of alleles tested in each locus: A ($n = 9$), B ($n = 22$) and DR ($n = 11$) using Bonferroni correction test.

RESULTS

Baseline characteristics of patients

The baseline clinical and virological characteristics of 23 patients who received IFN-α2b plus RBV combination therapy after LDLT are summarized in Table 2. All patients lacked evidence of coinfection with hepatitis B virus (HBV) or human immunodeficiency virus before LDLT. Of 23 patients, 20 (87.0%) were infected with HCV genotype 1b. Before treatment, all patients had serum HCV RNA levels over 100 kIU/mL by the Amplicor HCV 2.0 assay. Moreover, HCV RNA levels were greater than 850 kIU/mL (ranging from 850 to 21 200 kIU/mL) in 17 cases (73.9%), which is equivalent to 8.5×10^5–2.12×10^7 copies/mL HCV RNA by RT-PCR (Roche) [16], indicating that a majority of the patients in this study had high baseline viral loads before treatment.

Of the 23 patients allocated to a 48-week schedule, 15 (65.2%) completed treatment and 8 (34.8%) withdrew from the treatment protocol because of adverse events, such as thrombocytopoenia. Of these eight patients, the median duration of IFN treatment, from initiation to withdrawal of

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Table 3 Predictive factors for SVR

	SVR (n = 7)	Non-SVR (n = 8)	P value
Age (years)*	54.9 ± 6.15	52.4 ± 8.80	0.685
Gender (male)	4 (57.1%)	4 (50.0%)	0.595
No fibrosis before treatment	1 (14.3%)	2 (25.0%)	0.554
ALT (IU/L)*	152.4 ± 119.2	178.6 ± 100.8	0.643
Prothrombin time (INR)*	1.07 ± 0.17	1.10 ± 0.16	0.563
Total bilirubin (mg/dL)*	0.84 ± 0.31	2.10 ± 3.39	0.685
Creatinine (mg/dL)*	0.76 ± 0.24	0.73 ± 0.17	0.862
Virus genotype non-1b	2 (28.6%)	1 (12.5%)	0.446
Actual virus loads†	953 (120–1960)	1040 (347–21200)	0.177
Virus loads (>850 kIU/mL)	5 (71.4%)	6 (75.0%)	0.662
Reduction of IFN dose	1 (14.3%)	1 (12.5%)	0.733
Reduction of RBV dose	3 (42.9%)	4 (50.0%)	0.595
IFN dosage (300 MU)	3 (42.9%)	3 (37.5%)	0.622
Disappearance of HCV RNA			
4 weeks	5 (71.4%)	0 (0%)	0.007‡
12 weeks	6 (85.7%)	3 (37.5%)	0.084
24 weeks	7 (100%)	3 (37.5%)	0.019‡
Decline of HCV RNA by 2 $\log_{10}$ in 2 weeks	6 (85.7%)	2 (25.0%)	0.032‡

Statistics are calculated using either Mann–Whitney *U*-test or Fisher exact test; SVR, sustained virological response; ALT, alanine aminoteransferase; IFN, interferon; RBV, ribavirin; MU, million units.
*Values are mean ± SD or median (range).
†Values are median (range).
‡Statistically significant difference ($P < 0.05$).

the combination therapy, was 4 weeks (ranging from 1 to 15 weeks). Of the 15 patients who completed the treatment protocol, dose modification of either IFN-α2b or RBV was required in seven patients (46.7%). Among them, two patients had a redactor of IFN-α2b dose from 6 to 3 MU because of neutropoenia and thrombocytopoenia. Nine patients had a decreased dose of RBV during the therapy for the progressive anaemia. After treatment was completed, median blood levels returned to normal within 8 weeks in all patients. No patients died during the study period.

Factors associated with a sustained virological response

Sustained virological response was achieved in 7 of the 23 patients (30.4%). As none of the eight patients who withdrew from the study achieved SVR, completion of the combination therapy was a predictive factor of SVR (P = 0.026 and <0.05). Next, to determine predictive factors for the efficiency of the combination treatment in the 15 patients who completed the combination therapy, we examined the correlation between response to antiviral therapy and various baseline factors (Table 3). Two of three patients (66.7%) with the HCV genotype non-1b achieved SVR, whereas 5 of the 20 patients (25.0%) with the HCV genotype 1b achieved SVR. Among the 15 patients who had a pretreatment viral titre over 850 kIU/mL and infected with genotype 1b, four (26.7%) achieved SVR, whereas one (20.0%) of the five patients with a viral titre below 850 kIU/mL and genotype 1b achieved SVR. In addition, there was no relationship between SVR and actual viral load in each case ($P = 0.177$). These results showed that SVR was not significantly related to the baseline viral load before treatment in these transplant recipients with genotype 1b.

We then analysed the time course of viral load during IFN-α2b plus RBV combination therapy after LDLT. All seven cases with SVR cleared serum HCV RNA at 24 weeks after combination therapy, whereas HCV RNA was undetectable in three of the eight nonresponsive (non-SVR) patients (37.5%) who completed the treatment. Moreover, HCV RNA was undetectable in five of the seven SVR patients (71.4%) at 4 weeks after the start of treatment, whereas all eight non-SVR patients were HCV-RNA-positive at the same period ($P < 0.05$). Consistent with the rate of HCV disappearance in sera, a 2 $\log_{10}$ decrease in HCV RNA was observed 2 weeks after combination therapy in six of the seven SVR patients, a significantly greater decline compared with that observed in non-SVR patients (two of the eight patients) ($P < 0.05$). There was no relationship between SVR and other baseline factors.

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Detection of defective HCV RNA in sera of post-LDLT patients with HCV recurrence

Hepatitis C virus genotype 1b and baseline high viral concentrations are independent factors associated with poor response to IFN-α2b plus RBV combination therapy [7,8]. In this study, a substantial number of patients with extremely high viral load (above 850 kIU/mL) obtained SVR with the combination treatment. We therefore assessed whether serum HCV RNA titre, as determined by the widely used quantitative RT-PCR assay using the primer set specific for the 5′-untranslated region (5′-UTR) of HCV RNA [17–19], reflected the actual viral activity in patients with extremely high viral titre (above 850 kIU/mL) and genotype 1b under immunosuppressive conditions after LDLT. To determine the viral population circulating in the sera of these patients, we investigated HCV clones in 15 patients with genotype 1b and a pretreatment viral load above 850 kIU/mL. To amplify the HCV genome, we designed various primer sets spanning the 5′-UTR and the nonstructural protein (NS) 3 regions (Fig. 1a). Using conventional 5′-UTR primers, we confirmed that the amplified signal derived from HCV RNA corresponded to the expected size in all the 15 patients (data not shown). In contrast, using the primer sets spanning from the core to the NS3 region (HCV-P1 and HCV-P2), HCV sequences were not amplified in three patients, suggesting the presence of nucleotide alterations in the region of the HCV genome corresponding to these primer sequences. We confirmed that the signal amplified by the HCV-P1 primer set corresponded to the expected size in the 12 patients, from whom HCV sequences were amplified. Notably, we also revealed sequences that were smaller than expected in the sera of four patients (patients 1, 2, 3 and 6), suggesting there was a deletion in the HCV genome. As shown in Fig. 1a, the expected size of HCV RNA amplified by the HCV-P1 primer set was 2919 bp. In addition to this fragment, however, other signals with a smaller sequence size were detected in patients 1, 2, 3 and 6 (Fig. 1b; data for patients 2 and 3 are shown).

Slippage can occur during the target amplification process of *Taq* DNA polymerase [20]. Therefore, it is possible the HCV

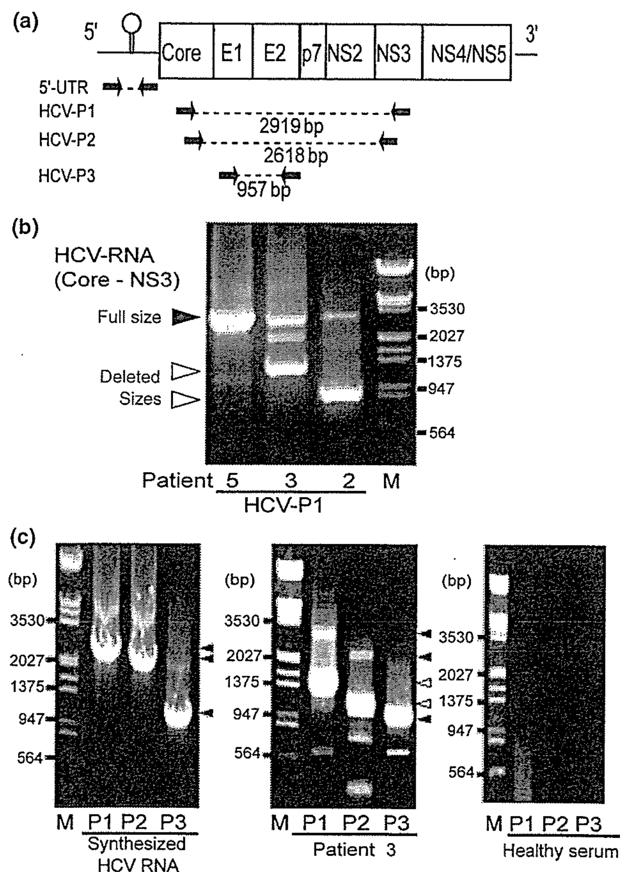

Fig. 1 Defective HCV clones detectable in the sera of highly viraemic patients with recurrent hepatitis C after LDLT. (a) Schematic presentation of the HCV genome and the primer sets used in this study. The sense and antisense primers specific for that part of the HCV sequence spanning 5′-UTR and NS3 are shown as arrows. The primer set specific for the 5′-UTR is generally used for the quantitative RT-PCR-based assay. Both HCV-P1 and HCV-P2 primer sets amplified the regions spanning from the core to the NS3 region and produced fragments of 2919 and 2618 bp in size, respectively. Amplification using the HCV-P3 primer set resulted in 957 bp fragments. 5′-UTR, 5′-untranslated region; E1, envelope glycoprotein-1; E2, envelope glycoprotein-2; NS, nonstructural protein. (b) Total RNA was extracted from the pretreatment sera of three patients (patients 2, 3 and 5). HCV RNA was determined by RT-PCR using the HCV-P1 primer sets. Black arrowheads indicate the fragment size of 2919 bp, corresponding to HCV RNA sequences of the expected size. Shorter fragments derived from HCV RNA amplification are indicated with white arrowheads. Lane M = λ DNA (Toyobo, Tokyo, Japan), digested with *Eco*RI and *Hin*dIII restriction enzymes, as a molecular weight marker. (c) The full-length HCV RNA genome was synthesized *in vitro* and used as a template in the RT-PCR assay as a positive control (left panel). Total RNA was prepared from the sera of patient 3 (middle panel) and a healthy individual without HCV infection (right panel). HCV RNA was amplified by RT-PCR using the primers HCV-P1, HCV-P2 and HCV-P3 and each RNA specimen as a template. Black arrowheads indicate expected sizes of fragments corresponding to the wild-type HCV generated by HCV-P1 (2919 bp, lane P1), HCV-P2 (2618 bp, lane P2) and HCV-P3 (957 bp, lane P3). Shorter fragments of 1374 bp (lane P1) and 963 (lane P2) bp are shown as white arrowheads (middle panel). Note that the HCV-P3 primer set only amplified the fragment corresponding to the wild-type clone with the expected size (957 bp) in patient 3. Lane M = λ DNA, digested with *Eco*RI and *Hin*dIII restriction enzymes, as a molecular weight marker.

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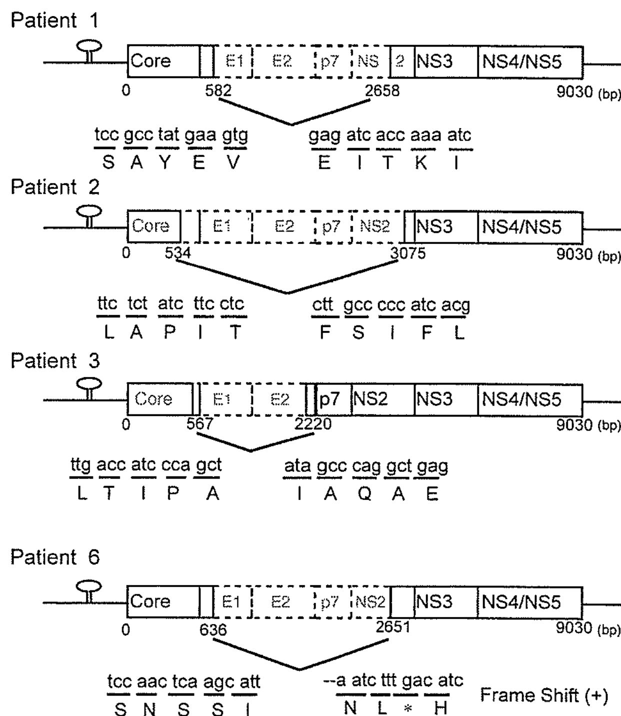
RNA deletion was due to an artefact during PCR amplification. To exclude this possibility, we amplified HCV sequences using several primer sets that spanned various regions spread over the HCV RNA genome in these patients (Fig. 1a). First, using *in vitro* synthesized full-length HCV RNA as a template, we confirmed that HCV fragments of the expected size (2919, 2618 and 957 bp) were amplified by the three different primer sets (HCV-P1, HCV-P2 and HCV-P3), respectively (Fig. 1c, left panel). Notably, we detected shorter HCV RNA fragments in the sera of transplant recipients with high viral loads. For example, using primer sets HCV-P1 and HCV-P2, short fragments of 1374 and 963 bp, respectively, as well as HCV RNA fragments of the expected sizes, were detected in the sera of patient 3 (Fig. 1c, middle panel). In contrast, the signals derived from the amplification using the primer set spanning the envelope region (HCV-P3) mainly included a fragment corresponding to wild-type HCV accompanied by a faint short signal. Taken together, these findings suggest that the shorter HCV RNA fragments were not an artefact of the PCR and might be derived from the sequences around the region between envelope glycoprotein 1 (E1) and NS2 in defective HCV clones.

To determine the internal structure of these deletions, all amplified fragments from four patients were subcloned for further sequence analyses. Schematic representations of defective HCV RNA detected in the sera of these patients are shown in Fig. 2. All defective clones identified had complete deletion of the E1 and envelope glycoprotein 2 (E2) regions of HCV RNA; that is, the shorter HCV clones detected in patients 1 and 6 contained the 3′-terminal part of the core, and the clones detected in patients 2 and 3 were also defective in the C-terminal of the core sequences. Nucleotide deletions in three (patients 1, 2 and 3) of four cases did not lead to a change in the reading frame, whereas the deletion in the coding sequence in patient 6 resulted in a change in the reading frame and thus a new stop codon was generated at the 886th amino acid. Although fragments of other sizes detected in these sera were also amplified by RT-PCR, sequence analyses revealed that these fragments were not identical to HCV RNA, suggesting that they were nonspecific PCR artefacts. To carry out quantitative analyses on the defective HCV clones, we performed northern blotting for HCV RNA using the extracted total RNA from sera as templates. However, signals derived from full and defective genomes were too weak to detect (data not shown).



Patient no.	HCV-RNA (KIU/L)	HCV clone GenbanK ID	Deleted region
1	850	AF165047	E1, E2, p7, NS2
2	1060	AF165058	Core, E1, E2, p7, NS2
3	2960	AF165058	Core, E1, E2
6	4020	AF207753	E1, E2, p7, NS2

Fig. 2 Schematic presentation of defective HCV clones in the sera of LDLT recipients with recurrent hepatitis C. The values in the schema indicate the nucleotide numbers from the first ATG of the core region in HCV RNA. Nucleotide and amino acid sequences immediately before and after the deleted region of the HCV genome are shown. The frame shift of the open reading frame occurred in the deleted HCV genome in patient 6; the changed amino acid sequence is shown under the codon, and the asterisk indicates a novel stop codon. E1, envelope glycoprotein-1; E2, envelope glycoprotein-2; NS, nonstructural protein.

Clinical significance of the defective HCV clones

Because a substantial number of post-LDLT patients with high viral loads had circulating defective HCV, we investigated the relationship between the presence of the defective HCV in sera and the various clinical factors in the 12 patients whose HCV RNA could be amplified by the HCV-P1 primer set (Table 4). Interestingly, none of the four patients with defective HCV RNA showed a 2 $\log_{10}$ decrease in HCV RNA within 2 weeks of the administration of combination therapy. In contrast, six of the eight patients (75.0%) whose sera contained only the wild-type HCV showed a prompt 2 $\log_{10}$ decrease in HCV RNA within 2 weeks of the administration of combination therapy ($P < 0.05$). Multivariate analysis, to assess whether the presence of defective clones significantly correlated with SVR, was difficult to carry out because of the limited number of patients. The patients without defective clones comprised three SVRs, two non-SVRs and three who withdrew from the study.

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Table 4 The association between the presence of HCV defective clones and the clinical factors

	Defective clones		
	Presence (n = 4)	Absence (n = 8)	P value
Age (years)*	56.3 ± 3.59	52.5 ± 7.21	0.396
Gender (male)	3 (75.0%)	4 (50.0%)	0.424
No fibrosis before treatment	0 (0%)	4 (50.0%)	0.141
ALT (IU/L)*	152.5 ± 128.3	271.0 ± 241.5	0.308
Prothrombin time (INR)*	1.05 ± 0.04	1.34 ± 0.72	0.497
Total bilirubin (mg/dL)*	0.85 ± 0.33	3.52 ± 5.00	0.552
Creatinine (mg/dL)*	0.80 ± 0.18	0.74 ± 0.20	0.497
IFN dosage (300 MU)	3 (75.0%)	5 (62.5%)	0.594
Decline of HCV RNA by 2 $\log_{10}$ in 2 weeks	0 (0%)	6 (75.0%)	0.030†
SVR of therapy	1 (25.0%)	3 (37.5%)	0.594

Statistics are calculated using either Mann–Whitney *U*-test or Fisher exact test. The data on 12 patients whose HCV RNA in the sera could be amplified by the primer sets spanning from the core to the non-structural protein 3 region are shown. ALT, alanine aminoteransferase; IFN, interferon; MU, million units; SVR, sustained virological response.
*Values are mean ± SD.
†Statistically significant difference ($P < 0.05$).

Although two patients lacking defective clones were defined as non-SVR, they also showed the 2 $\log_{10}$ decline in HCV RNA within 2 weeks of the administration of combination therapy and the disappearance of HCV RNA during the IFN-α2b plus RBV therapy. The patients with defective clones comprised one SVR, two non-SVRs and one who withdrew from the study. The SVR patient, in whom the defective HCV was detected before treatment, had a slow decline in viral titre, and disappearance of HCV RNA was achieved at 24 weeks of therapy.

DISCUSSION

To date, IFN-α2b plus RBV combination therapy has become one of the gold standards for the treatment of recurrent hepatitis C following liver transplantation [6,21,22]. However, little is known about factors that predict the efficacy of the combination therapy in posttransplant patients with extremely high viral load under immunosuppressive conditions. In this study, we have demonstrated that combination treatment with IFN-α2b plus RBV induced SVR in 30.4% of patients with recurrent hepatitis C after LDLT. This SVR rate was similar to the data from previous studies of ORLT patients [9,10]. We also revealed that a 2 $\log_{10}$ decline in HCV RNA levels within 2 weeks after starting the treatment and the clearance of HCV RNA at 4 or 24 weeks after the start of treatment was predictive for SVR in these patients. Our findings are in agreement with previous studies showing that the early decline of HCV RNA following the start of combination therapy is a predictive factor for SVR in patients with HCV-induced chronic liver disease without transplantation [23,24].

Hepatitis C virus reinfection in posttransplant patients differs from HCV infection in immunocompetent nontransplant patients in several aspects. One of the most striking features of transplant recipients with recurrent hepatitis C is their extremely high viral load as a result of immunosuppressive conditions [6]. In fact, most patients examined in this study had pretreatment viral titres over 850 kIU/mL; this extremely high viral load is rarely observed in immunocompetent patients with chronic HCV infection. High viral load and viral genotype 1b are widely accepted as predictors of unresponsiveness to antiviral therapy in immunocompetent patients with chronic HCV infection [7,8]. In contrast, we found that pretreatment viral titre had no influence on the efficacy of the combination therapy in transplant recipients under immunosuppressive conditions, and a considerable number of patients with a high viral load of genotype 1b HCV underwent an early decline in HCV RNA and achieved SVR after the administration of IFN-α2b plus RBV. These findings suggest the presence of other host or viral factors that might influence responsiveness to IFN-α2b plus RBV combination therapy in highly viraemic patients under immunosuppressive conditions.

Interestingly, when assessing viral factors that might influence responsiveness to antiviral treatment, we found that novel defective HCV clones that contain large deletions in the viral genome are present in the serum of recurrent hepatitis C patients with high viral load after LDLT. These defective clones occur widely in both RNA and DNA viruses

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in bacteria, plants and animals [25–31]. In particular, RNA viruses have the ability to change rapidly and frequently generate not only new strains but also defective interfering RNAs [32].

Although the precise reason for the development of such defective HCV RNA in patients with recurrent hepatitis C after LDLT is unclear at present, an impaired host immune response as a result of immunosuppressive treatment might somehow be involved in the appearance of these defective strains in transplant recipients. Similar findings have been reported in HBV-related transplant recipients, in which precore defective HBV clones occur in the sera during fulminant reinfection after liver transplantation [33]. Further analyses are required to clarify the relationship between the host immunity and the presence of defective HCV clones.

Few reports have identified defective HCV in patients with HCV infection. Yeh *et al.* [34] detected defective HCV with a truncated core protein in the ascites of a patient with HCC in association with intact HCV in the sera of the same patient. Another study has reported that sequences of the HCV core region in the liver tissues of HCC patients have some nucleotide deletions and mutations that result in a truncated core protein [35]. In contrast to these reports, all defective HCV clones detected in transplant recipients examined in this study had deletions in the large region spanning E1 and E2. It is unclear whether these defective viral clones are capable of replicating in hepatocytes. In tombusvirus, incomplete clones cannot replicate by themselves; however, their replication is rescued by the concurrent presence of homologous helper viruses [25]. In this regard, all of our patients with defective HCV RNA clones also had full-length HCV RNA in their sera. Accordingly, it is possible that coexisting full-length HCV serves as a helper sequence, enabling the encapsulation and secretion of the defective viral genome.

An important finding of this study is that six of the eight patients who lacked evidence of circulating defective virus showed a prompt decline in serum HCV RNA in response to antiviral therapy, whereas all patients with the defective strains responded poorly to IFN-α2b plus RBV combination therapy. When we investigated the relationship between the presence of defective HCV and the virological response to IFN therapy in immunocompetent patients with chronic hepatitis C, non-SVR cases were more likely to have defective clones than SVR cases; however, the influence of the defective clones did not reach statistical significance (unpublished data). Thus, further analyses are required to assess the possible role of defective HCV clones in immunocompetent patients with chronic hepatitis C. The reason for this remarkable difference in response to antiviral therapy between patients with and without defective clones remains to be determined; however, it may be reasonable to speculate that the defective strains modulate the course of the disease by affecting the activity of wild-type viruses, as many defective interfering viral RNA can compete with the helper virus during replication or during other steps in the infectious cycle.

In conclusion, IFN-α2b plus RBV can achieve SVR in one third of patients with recurrent hepatitis C after LDLT, despite infection with genotype 1b and a very high viral load. This work provides the first evidence that defective HCV RNA is present in the sera of some of highly viraemic patients under immunosuppressive conditions after liver transplantation, which may affect viral response to IFN-α2b plus RBV combination therapy. Further studies are required to assess the clinical significance of these defective HCV clones.

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ORIGINAL ARTICLE

Classification of Human Liver Transplant Recipients by their Preoperative CD8$^+$ T Cell Subpopulation and its Relation to Outcome

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The primed status of T cells is markedly different among liver transplant recipients, due to a lifetime of antigen exposure and reduced thymopoiesis by aging, and diseases. This study aims to characterize the preoperative immunological status of CD8$^+$ T cell subpopulations and relate it to the outcome for liver transplant recipients. We classified 112 liver transplant recipients into 5 groups, based on hierarchical clustering of the CD8$^+$CD45 isoform proportion of T cells. In Groups I and II (pediatric), the naive T cell proportion was more than 50%. In adult recipients, Group III was characterized by a naive T cell proportion of 50%, Group IV had the greatest effector/memory T cells (EM), and Group V had the greatest proportion of effector T cells. In Groups IV and V, the effector T cell proportion was considerably higher, and was accompanied by marked downregulation of the CD27$^+$CD28$^+$ subsets and upregulation of interferon gamma (IFN)-γ, tumor necrosis factor-alpha, and perforin expression. Group V recipients tended to be complicated postoperatively, with a significantly reduced survival rate (1 yr, 66.8%) and markedly reduced Eastern Cooperative Oncology Group performance status. *Liver Transpl 12:792-800, 2006.*
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Following liver transplantation, various factors have been found to contribute to graft dysfunction, including etiology of liver disease, recipient and donor age,[1] and retransplantation, tissue damage during organ retrieval, storage and surgery, and insufficient hepatic mass.[2] Immunity after organ transplantation involves a continuing battle, with chronic stimulation by various persisting antigens such as alloantigen and infections. After liver transplantation, large numbers of donor dendritic cells migrate to the recipients' secondary lymphoid tissues, notably the spleen and lymphoid organs—sites where naive T cells react with alloantigen-loaded dendritic cells and differentiate into effector and memory T cells. However, the postoperative alloreactive response is greatly affected by the immunological status of recipients prior to transplantation, itself related to age and disease status. Aging increases the proportion of memory phenotype T cells, probably reflecting cumulative exposure to environmental antigens and to a reduced thymic T cell supply.[3] The proportion of memory phenotype T cells may be affected by morbidities such as chronic viral infection and other bacterial and fungous infections. After transplantation,

Abbreviations: LDLT, living donor liver transplantation; HBV, hepatitis B virus; HCV, hepatitis C virus; FITC, fluorescein isothiocyanate; PE, phycoerythrin; CCR7, chemokine receptor 7; IFN-γ, interferon-gamma; naive T cell subsets, CD45RO$^-$CCR7$^+$; CM, central/memory T cell subsets, CD45RO$^+$CCR7$^+$; EM, effector/memory T cell subsets, CD45RO$^+$CCR7$^-$; effector T cell subsets, CD45RO$^-$CCR7$^-$; CTL, cytotoxic T lymphocyte; HLA, human leukocyte antigen.
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immunosuppression can modify infectious pathogenic pathways. In particular, postoperative infection is still the most common cause of death in liver transplant recipients. In host defense, infected cells are eliminated by cytotoxic $CD8^+$ T cells, with help from the CD4 helper T cells.[4,5] The chemokine receptor CCR7 controls homing of $CD8^+$ T cells to secondary lymphoid organs, and divides human memory T cells into 3 functionally distinct subsets: central/memory (CM), effector/memory (EM), and effector T cells.[6] Also, expression of the costimulatory receptors CD28 and CD27 is associated with different stages of $CD8^+$ T cell differentiation in persistent virus infection in humans.[7] We therefore set out to identify the immune risk phenotype corresponding to mortality according to the degree of $CD8^+$ T cell cytotoxic activity along which sequential downregulation of the CCR7 and $CD27^+CD28^+$ subsets occurs, accompanied by upregulation of cytotoxic factors.

Living donor liver transplantation (LDLT) is an established therapeutic modality for children and adults with end-stage liver disease. At Kyoto University we began a pediatric LDLT program in 1990[8-10] and an adult program in 1998.[11-13] By August 2004 we had performed more than 1,000 LDLTs. The indications for LDLT have been expanded to include terminal liver disease associated with hepatitis C or B virus (HCV or HBV) infection in adults. HCV-associated end-stage liver disease has recently become one of the most common indications in adult LDLT series.[14] Although HBV has been overcome by antiviral treatments, HCV infection in immunosuppressed transplant recipients usually leads to faster and more severe HCV recurrence, in contrast to immunocompetent individuals;[15,16] it progresses to significant fibrosis and approximately 30% cirrhosis at 5 yr after liver transplantation.[17]

The present study aims to identify any characteristics of the pretransplantation $CD8^+$ subpopulation—the immune risk phenotype—that correlate with postoperative complications in heterogeneous recipients.

PATIENTS AND METHODS

Patients and Graft

The study involved 112 subjects who had undergone standard LDLT between 2002 and 2005 at Kyoto University Hospital. Their ages ranged from 1 month to 67 yr. The patients were followed up from the time of transplantation until July 2005 or death. The median follow-up period was 1.9 yr after LDLT (range: 7 months to 2.8 yr). No patients received antiviral therapy after LDLT. HCV- and HBV-positive donors were not used. Written informed consent was obtained before the start of the study, which was approved by the Ethics Committee of Kyoto University Hospital and was conducted in accordance with the Declaration of Helsinki of 1975 as revised in 1996.

Immunosuppression

All patients underwent standard LDLT.[18] The initial immunosuppression regime after LDLT was tacrolimus or cyclosporin with corticosteroids. Administration of tacrolimus (1.5 mg by mouth every 12 hours) was begun on the evening of the day after the operation, according to our standard procedure.[19] Doses were adjusted according to the whole-blood trough concentration, which was measured daily about 12 hours after the evening dose. The target whole-blood trough level was between 10 and 15 ng/mL during the first 3 weeks and approximately 10 ng/mL at the end of the first month; in the outpatient stage it was maintained between 5 and 10 ng/mL. The initial dose of steroids was reduced rapidly, and was withdrawn totally by 3-6 months after transplantation. Methylprednisolone (1 mg/kg) was given intravenously every 12 hours for 3 days, starting on the day of surgery, after which prednisone (0.5 mg/kg) was given for 3 days. On day 7 the prednisone was reduced to a daily maintenance level of 0.3 mg/kg, given orally.

Definition and Treatment of Acute Graft Rejection

In cases of clinical or laboratory signs of acute graft rejection, a liver biopsy was performed percutaneously. The specimens were graded according to the Banff criteria[20] as mild, moderate, or severe graft rejection. After diagnosis of acute graft rejection was confirmed, patients received a 3- to 5-day course of intravenous steroid bolus therapy (10 mg/kg). Graft loss was defined by death or retransplantation.

Definition of Infectious Complication

A bacterial, viral, or fungal infection was assumed if clinical or laboratory signs of acute infection or positive serologic markers or culture were found. In proven cases of infection, the treatment followed general recommendations.

Virology Assay

Serum qualitative HCV-ribonucleic acid was determined by the polymerase chain reaction method using a commercially available assay (Amplicor HCV; Roche Molecular Systems, Pleasanton, CA). The HCV genotype was determined with a system based on polymerase chain reaction using genotype-specific primers.[21] In the 26 recipients assayed, 25 had pretransplantation HCV 1b genotype.

Tissue Typing

Serologic tissue typing was performed in all patients for human leukocyte antigen (HLA)-A, HLA-B (Bw), HLA-C, HLA-DR, and HLA-DQ loci.

Flow Cytometry

In recent years, major histocompatibility complex class I tetramer technology has been applied to clarify the

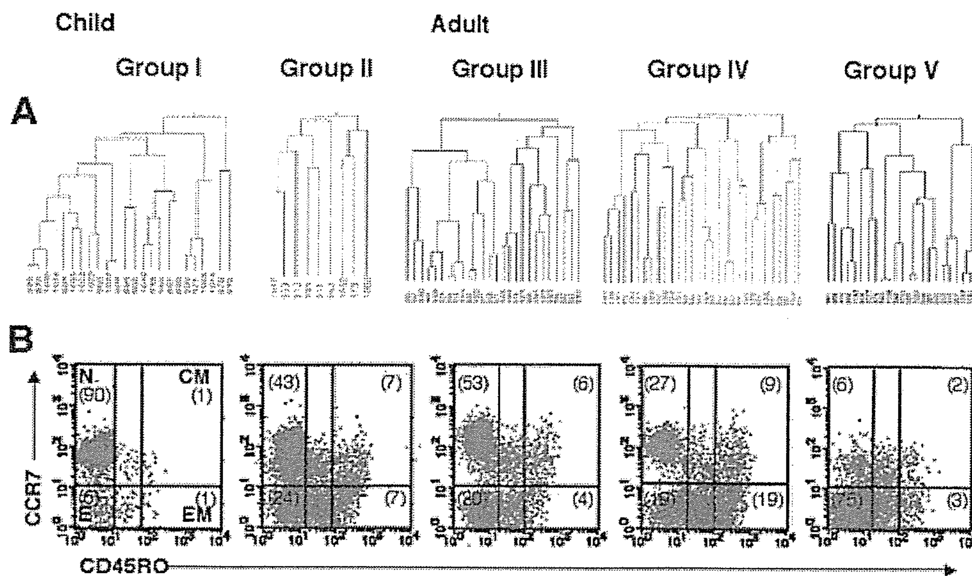

Figure 1. (A) Dendrogram of hierarchical clustering. (B) CD45RO and CCR7 are coexpressed on the subsets of peripheral blood CD8$^+$ T cells. Lymphocytes were stained with monoclonal antibodies to CD45RO and CCR7, which identified 4 subsets of CD8$^+$: 1 naive (N) (CD45RO$^-$CCR7$^+$); and 3 memory subsets, CM (CD45RO$^+$CCR7$^+$), EM (CD45RO$^+$CCR7$^-$), and effector T cells (E) (CD45RO$^-$CCR7$^-$). Percentages of cells in each subset are shown.

surface phenotype and functions of antigen-specific CD8$^+$ T cells. However, our study aims to clarify the phenotypic and functional changes of the CD8$^+$ subpopulation in many recipients (with or without viral infection) classified according to CD8$^+$CD45 isoform profiles prior to LDLT. Analysis of global nonspecific CD8$^+$ T cells was therefore used to follow up the relation of CD8$^+$ T cell function to the clinical outcome.

Heparinized venous blood samples were obtained 1 hour prior to surgery and then at 0, 1, 3, 6, 12, 36, and 120 hours, and every week following graft reperfusion for 4 months. As control samples, venous peripheral blood was collected from 54 healthy laboratory personal and medical students (32 men, 22 women; mean age 30 ± 3 yr standard error, range 4-69 yr). Since CD45RA and CD45RO expression are mutually exclusive we measured only the CD45RO isoform, but we used CD45RA for measuring cytokine. Naive T cells were defined as CD45RO$^-$CCR7$^+$; central/memory (CM) T cells as CD45RO$^+$CCR7$^+$; effector/memory (EM) T cells as CD45RO$^+$CCR7$^-$; and effector T cells as CD45RO$^-$CCR7$^-$.[6]

The monoclonal antibodies used to stain cell surface antigens were as follows: allophycocyanin (Coulter Immunotech, Miami, FL) or phycoerythrin-cyanin-5-conjugated (Coulter Immunotech, Marseilles, France) anti-CD4 or CD8, fluorescein isothiocyanate (FITC)-conjugated anti-CD45RO (Nichirei, Tokyo, Japan), TRI-COLOR-conjugated anti-CD45RA (Caltag Laboratories, Burlingame, CA), phycoerythrin (PE)-conjugated anti-CD3 (Coulter Immunotech, Miami, FL), FITC-conjugated anti-CD19 (Coulter Immunotech, Marseilles, France), PE-conjugated anti-human CCR7 (DakoCytomation, Kyoto, Tokyo, Japan), PE-conjugated anti-CD27 (Coulter Immunotech, Marseilles, France), and FITC-conjugated anti-CD28 (Nichirei, Tokyo, Japan). We used isotype-matched controls for intracellular staining. Cells were exposed to the antibodies for 30 minutes at 4°C and were washed twice with phosphate buffered saline; 5,000 cells were analyzed. FITC- and PE-labeled mouse immunoglobulin G were used as isotype-matched background controls. We analyzed the stained cells on a fluorescence-activated cell sorter Calibur flow cytometer by 3- and 4-color analysis, using CELL Quest software version 3.3 (BD Biosciences, San Jose, CA).

Flow Cytometric Detection of Cytokine Production and Intracellular Staining for Perforin

Flow cytometric measurement of cytokine production was performed as described previously.[22] In summary, 10^6 cells/mL were stimulated for 4 hours (interferon-gamma, IFN-γ, FITC-conjugated anti-IFN-γ, Becton Dickinson, San Jose, CA; tumor necrosis factor-alpha, tumor necrosis factor-alpha, FITC-conjugated anti-tumor necrosis factor-alpha, BD Bioscience, San Diego, CA) with a mixture of phorbol 12-myristate 13-acetate (25 ng/mL; Sigma-Aldrich Chemical, St. Louis, MO) and ionomycin (1 μg/mL; Sigma-Aldrich). The Golgi inhibitor brefeldin A (10 μg/mL; Sigma-Aldrich) was added for retention of intracellular cytokines. The cells were then stained for surface markers with PE, phycoerythrin-cyanin-5 (or TRI-COLOR), and allophycocyanin-conjugated antibodies, permeabilized with fluorescence-activated cell sorter lysing solution and fluorescence-activated cell sorter permeabilizing solution (BD Biosciences, San Diego, CA), and then stained for the indicated intracellular cytokines with FITC or PE-conjugated antibodies.

We measured intracellular perforin in CD8$^+$ cells without previous stimulation, and used the permeabilization and staining protocol described above for cytokine analysis. For the perforin analysis, the cells were treated with fixing buffer (Caltag Laboratories, Austria) for 20 minutes at room temperature, washed with phosphate buffered saline-0.1% fetal calf serum, and permeabilized with a permeabilization buffer (Caltag Laboratories, Austria) for 20 minutes at room temper-

TABLE 1. Hierarchical Clustering into 5 Groups

Group	n	Age (yr)	% Naive T cells	% CM T cells	% EM T cells	% Effector T cells	% $CD27^+CD28^+$ subsets	% IFN-γ	% TNF-α	% Perforin
Child										
I	24	3 ± 3	84.91 ± 7.54	0.85 ± 0.85	1.65 ± 2.10	7.98 ± 4.36	83.19 ± 7.26	0.84 ± 0.71	0.25 ± 0.37	3.61 ± 4.32
II	9	7 ± 5	44.95 ± 11.70	1.52 ± 3.53	8.93 ± 7.50	32.03 ± 8.44	59.97 ± 15.82	8.82 ± 3.99	4.28 ± 1.17	19.65 ± 7.29
Adult										
III	26	46 ± 11	53.66 ± 8.14	6.31 ± 3.47	7.70 ± 6.60	18.60 ± 7.93	70.62 ± 8.76	9.74 ± 5.38	9.70 ± 6.10	15.68 ± 6.68
IV	30	55 ± 9	19.85 ± 11.14	12.33 ± 5.55	18.10 ± 10.31	27.83 ± 12.82	44.23 ± 17.37	13.43 ± 8.57	13.32 ± 7.23	24.33 ± 11.46
V	23	49 ± 13	23.43 ± 10.75	3.73 ± 1.89	8.57 ± 5.40	46.71 ± 14.22	46.48 ± 17.10	27.58 ± 13.71	24.92 ± 16.25	32.11 ± 14.06

NOTE: Values are expressed as mean ± SD.

ature. They were then stained with antiperforin (δG9) (BD PharMingen, Crowley, UK) followed by addition of R-PE-CY5-conjugated $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulin (DakoCytomation) secondary antibody.

Statistical Analysis

We classified recipients into 2 groups: pediatric (under 18 yr of age) and adult. We performed hierarchical cluster analysis in both groups, using JMP 5 (SAS Institute, Cary, NC)[23] to obtain clusters of recipients having similar proportions of naive, CM, EM, and effector T cells.

We determined bivariate correlations by Spearman rank correlation. Comparisons for continuous variables between groups were performed using Student's *t*-test and analysis of variance. Comparisons for proportions between groups were performed using Fisher's exact test. Survival curves were estimated using the Kaplan-Meier method, and log-rank tests were applied to test associations between group and survival time. All statistical tests were 2-sided, with significance defined as $P < 0.05$. Statistical analyses were performed using the statistical software package StatView 5 (Abacus Concepts, Berkeley, CA).

RESULTS

Hierarchical Clustering by Preoperative $CD8^+CD45$ Isoform Profiles

The existence of 5 groups, classified according to hierarchical clustering of our 112 recipients, was clear, as seen in the dendrogram (Fig. 1A). CD45RO and CCR7 were coexpressed on a subset of peripheral blood $CD8^+$ T cells in a typical recipient of each group (Fig. 1B). The proportion of cells in the different compartments was reasonably stable in the same group, but more variable across the 5 groups. In pediatric recipients the pretransplantation mean proportion of naive T cells was 85% in Group I and 45% in Group II; the effector T cell population was only marginal in Group I, but was high in Group II (Table 1). In adults, the naive T cell population was considerably lower in Groups IV and V than in Group III. The $CD8^+$ T cells in Group IV included the greatest number of EM T cells, and in Group V included the greatest number of effector T cells. In Groups IV and V the proportion of IFN-γ, tumor necrosis factor-alpha and perforin expression were markedly higher than in Groups I, II, and III. Table 2 shows statistical differences between the 5 groups in their proportions of the $CD8^+$ T cell subpopulation and their function. There were significantly large differences in CD45 isoforms between the 5 groups. In particular, the effector T cell proportion in Group V was significantly higher than in Groups III and IV. The proportion of IFN-γ differed significantly between Group IV and V recipients; tumor necrosis factor-alpha and perforin expression did not differ.

Table 3 profiles the recipients and donors. The study group included 53 recipients who underwent LDLT for

TABLE 2. Results of *P*-values Presenting Differences Among 5 Groups in the Relative Proportion of CD8$^+$ T Cell Subpopulation

	Child	Adult			
Variable	Group I vs. II P^*	$P^\dagger$	Group III vs. IV P^*	Group III vs. V P^*	Group IV vs. V P^*
Age (yr)	0.0577	0.0148	0.0021	0.2857	0.1035
% Naive T cells	<0.0001	<0.0001	<0.0001	<0.0001	0.2449
% CM T cells	0.0070	<0.0001	<0.0001	0.0027	<0.0001
% EM T cells	0.0005	<0.0001	<0.0001	0.6171	0.0002
% Effector T cells	<0.0001	<0.0001	0.0025	<0.0001	<0.0001
% CD27$^+$CD28$^+$ subsets	<0.0001	<0.0001	<0.0001	<0.0001	0.6819
% IFN-γ	0.0061	0.0003	0.1847	0.0002	0.0163
% TNF-α	<0.0001	0.0058	0.1571	0.0043	0.0748
% Perforin	<0.0001	<0.0001	0.0092	<0.0001	0.0552

Abbreviations: ANOVA, analysis of variance; CM, central/memory T cell subsets, EM, effector/memory T cell subsets.
*P-values are based on Student's *t*-test.
†P-values are based on ANOVA.

chronic HCV or HBV infection. One Group IV recipient was coinfected simultaneously with HCV and HBV, and was involved in the HCV and HBV groups. The majority of Group III (53.8%), Group IV (76.7%), and Group V (69.6%) recipients suffered from chronic HCV- and/or HBV-infection. HCC was more prevalent in Groups IV and V (approximately 70%) than in Group III (36%). The 3 adult groups did not differ significantly in clinical status according to the Model for End-Stage Liver Disease score.[24] ABO blood group-incompatible LDLT was carried out in 3 children and 15 adults. The adult recipients did not differ in the amount of liver tissue transplanted, but the graft-to-recipient weight ratio in the adult groups was only about 0.52 times the ratio in the younger groups. There were significantly more HLA mismatched loci in Group V than in Groups I and III. The duration of cold ischemia was slightly longer in Group IV and V recipients.

Figure 2 shows changes of the effector T cell proportion in circulating CD8$^+$ T cells with advancing age in 112 transplant recipients and 54 healthy individuals. There was no correlation (r = 0.39) between the effector T cell proportion and advancing age in healthy individuals, but a weak correlation (r = 0.55) was found in the recipients. In the healthy individuals, the proportion of effector T cells was lower (13.71 ± 1.92, mean ± standard error) in pediatric recipients (under 18 yr of age) than in adult recipients (30.19 ± 1.83); $P < 0.001$. In adult recipients there was no significant difference in the effector T cell proportion between healthy individuals and Group III or IV recipients ($P = 0.14$ and $P = 0.07$, respectively). In contrast, the difference between healthy individuals and Group V recipients was significant ($P < 0.0001$). The proportion of effector T cells was considerably higher in Group IV (13%) and Group V (44%) than the upper limits for healthy individuals.

Postoperative Complication in the 5 Groups

Figure 3 (left) shows Kaplan-Meier curves for the recipient's probability of survival in the 5 groups. The 2-yr survival was 96% in Group I, 89% in Group II, 100% in Group III, 89% in Group IV, and 74% in Group V (Group V vs. Group III, $P < 0.01$). The Eastern Cooperative Oncology Group performance status[25] was assessed objectively for surviving patients up to 1 yr after LDLT. The proportion of recipients with Grade 0 Eastern Cooperative Oncology Group performance status (fully active and able to carry on all predisease activities without restriction) decreased progressively from Group I to Group V, in which it was only 9% (Fig. 3, right). Table 4 shows the frequencies of rejection and infection in the 5 groups. Rejection frequencies were higher in pediatric recipients than in adults. The phenotypic and functional profiles prior to LDLT in pediatric Group II were quite similar to those of adult Group III. However, the incidence of rejection tended to be higher in Group II than in Group III ($P = 0.112$, Fisher's exact test). In Group II recipients, the CD8$^+$naive T cell proportion prior to LDLT was low compared with that in Group I, but promptly upregulated to high levels, similar to Group I, following tacrolimus administration 24 hours after LDLT; there was corresponding downregulation of effector T cells and cytolytic activity (data not shown). This rapid restoration of naive T cells seems to depend on intact thymic function during early life. In adult Croup III, in contrast, the naive T cells could not be restored to high levels because of the involvement of the thymus by advancing age. It is not clear why CD4$^+$ and CD8$^+$ T cells with high levels of naive T cells are more closely related to rejection than T cells with lower naive T cells. It is likely that the incidence of rejection in Group II are similar to those of Group I, but are higher than in Group III. On the other hand, there was no significant ($P = 0.686$, Fisher's exact test) difference in infection rate between Groups II and III. Group V recipients clearly had a higher infection rate than Group III.

Donor age has been reported to be an important factor affecting the severity of liver disease following liver transplantation.[1] Specifically, an adverse effect has been reported of advanced donor age (>40 yr) on the

TABLE 3. Recipient, Donor, and Operation Profiles

Recipients Group (n) male/female	Original liver diseases (n)	MELD score	Donor source (n)	HLA mismatch (n)	ABO blood type combination	Operation profiles Ischemic time (minutes)*	
						Cold	Warm
Group I (24) 9/15	BA (17), Byler (1), Alagille syndrome (1) hepatoblastoma (2), FHF (1), primary hyperoxaluria (1), tyrosinemia (1)	—	Parent (23), uncle (1)	1 (5), 2 (10), 3 (4)	Identical (16), compatible (6), incompatible (2)	89 ± 55	53 ± 26
Group II (9) 5/4	BA (4), chronic rejection (3, BA), hepatoblastoma (1), FHF (1)	—	Parent (8), aunt (1)	1 (3), 2 (3), 3 (1), 5 (1)	Identical (5), compatible (3), incompatible (1)	83 ± 117	40 ± 10
Group III (26) 12/14	PBC (3), PSC (2), FHF (6), polycystic disease (1), HBV (6 with 2 HCC), HCV (8 with 3 HCC)	13 ± 6	Parent (2), offspring (6), Spouses (7), sibling (10), cousin (1)	0 (4), 1 (4), 2 (4), 3 (5), 4 (4)	Identical (17), compatible (5) incompatible (4)	76 ± 51	51 ± 19
Group IV (30) 21/9	PBC (4), Alcholic LC (1), AIH (1), Caroli (1), HBV (10 with 6 HCC), HCV (12 with 10 HCC) HBV + HCV with HCC (1)	13 ± 6	Parent (1), offspring (15), spouses (7), sibling (6), nephew (1)	0 (2), 2 (12), 3 (7), 4 (1), 5 (1)	Identical (23), compatible (5), incompatible (2)	115 ± 64	68 ± 55
Group V (23) 11/12	BA (2), PBC (2), Wilson's disease (1), alcholic LC (1), polycystic disease (1) HBV (5 with 4 HCC), HCV (11 with 7 HCC)	16 ± 11	Parent (3), offspring (6), spouses (6), sibling (7), nephew (1)	0 (1), 2 (4), 3 (5), 4 (2), 5 (4), 6 (1)	Identical (11), compatible (3), incompatible (9)	133 ± 87	56 ± 21

Abbreviations: BA, biliary atresia; FHF, fulminant hepatic failure; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; LC, liver cirrhosis; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; AIH, autoimmune disease.
*Values expressed as mean ± SD.

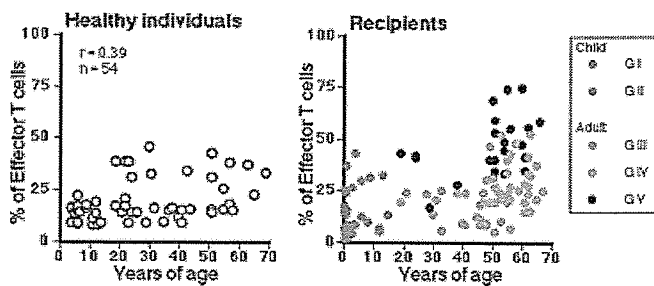

Figure 2. Changes in the proportion of circulating effector T cells with age in healthy individuals and transplant recipients. Each group is specified by a color marker: red, Group I; green, Group II; blue, Group III; brown, Group IV; and black, Group V.

outcome of transplantation for HCV.[26-30] In the present study, the mortality rates related to donor age were 8.6% (5/58) under 40 yr and 11.1% (6/54) over 40 yr ($P = 0.756$, Fisher's exact test).

During the study period, 11 (9.8%) of our 112 recipients died. Two of these were pediatric recipients; 1 suffered fulminant hepatic failure due to de novo autoimmune hepatitis, and the other suffered biliary atresia with retransplantation due to acute rejection. Nine adult recipients died (6 with either HCV or HBV infection, and 1 each with primary biliary cirrhosis, biliary atresia, and polycystic liver). Of 18 ABO blood group-incompatible LDLT, 3 (16.7%) died. The median age of the recipients was 48 yr (range, 1 month to 67 yr). The median age of donors was 38 yr (range, 21 to 64 yr). The median time from LDLT to death was 65 days (range, 13 to 351 days). Two recipients underwent retransplantation. Four of the deceased recipients were complicated by acute cellular rejection.

DISCUSSION

In Group I and II recipients, the survival probability was high and the Eastern Cooperative Oncology Group performance status was very good, but acute rejection occurred in approximately 60% (Fig. 3; Table 4). In adult recipients, in contrast, postoperative complications increased progressively from Group III to Group V. More postoperative complications developed in Group V recipients, leading to significant reduction in the survival probability and markedly reduced Eastern Cooperative Oncology Group performance status. These recipients were compromised by a high rate of life-threatening infection, rather than acute rejection.

Enhancement of $CD8^+$ Cytolytic Activity and Cytokine Production in Group IV and V Recipients

The outcome of the infection depends on how effectively the defensive mechanisms of the host resist the offensive tactics of the bacteria and virus.[31] In the present study, circulating $CD8^+$ T cells with a $CD45RA^+$-$CCR7^-$, combined with marked downregulation of $CD27^+CD28^+$, resembled cytolytic effector T cells. We have found that the interleukin-12 receptor $\beta 1$ subunit in $CD8^+$ T cells upregulates positively with the proportion of effector T cells and IFN-γ-producing cells immediately after LDLT in Group IV and V recipients (data not shown). It is possible that interleukin-12 is important in promoting Th1-type immune response and cytotoxic T lymphocyte (CTL) activity after LDLT. Moreover, the preferential increase of effector T cells in Group IV and V was accompanied by marked up-regulation of IFN-γ and tumor necrosis factor-alpha (Table 1). Their enhanced expression of perforin plays a critical role in this cytolytic effector, since it can polymerize to form channel-like structures in the target cell membrane, through which granzymes can enter and subsequently activate the death machinery.[32,33] We found here that granule exocytosis by perforin is already operational in circulating effector-type $CD8^+$ cells prior to LDLT. More importantly, $CD27^+CD28^+$ expression was used to distinguish between subsets of differentiated $CD8^+$ T cells at different stages immediately after LDLT. These subsets can be assigned a position on a $CD8^+$ T cell differentiation pathway along which sequential downregulation of $CD27^+CD28^+$ subsets occurs, accompanied by upregulation of cytotoxic factors. Downregulation of the levels of $CD27^+CD28^+$ subsets therefore indicates that the activity of CTLs in eliminating virus-infected self-cells increases progressively from Group III to Group V recipients prior to LDLT. Group III recipients were able to mount an immune response that might help to clear HCV-ribonucleic acid even during immunosuppressive therapy, probably involving sustained viral clearance irrespective of small increases in IFN-γ. In Group V recipients, in contrast, the high effector T cell proportion is probably associated with the greatly enhanced cytotoxic activity, but could not adequately eliminate viral-infected cells.

Viral-infected recipients were characterized in the present study by enrichment of $CD8^+$ T cells having differing phenotypes between groups during chronic infection. These differences in $CD8^+$ T cell phenotype may relate simply to the differential properties necessary to control a virus. The virus load increases at least 10-fold after liver transplantation,[34] so that such viral replication may contribute further to the development and maintenance of the increased effector T cell proportion after LDLT. Therefore, when there is high HCV messenger ribonucleic acid, the enhanced cytotoxic activity may relate to the high viral load, leading to marked suppression of the host-effector immune response that usually controls HCV replication.

Of the 9 deceased adult recipients, 6 had chronic or HCV or HBV infection and the remaining 3 had other diseases. It follows that the recipient's immune response, characterized by a high effector T cell population, is not specific for chronic viral infection, and apparently plays a critical role in controlling not only liver damage but also infections such as fungi and bacteria. The immunosuppressive cascade would also have a greater and catastrophic effect on these recipients. Importantly, there were marked differences in clinical outcome and CTL generation according to the $CD8^+$ naive T cell proportion prior to LDLT. In Groups I, II, and III, $CD8^+$ T cells with a high naive T cell proportion had low

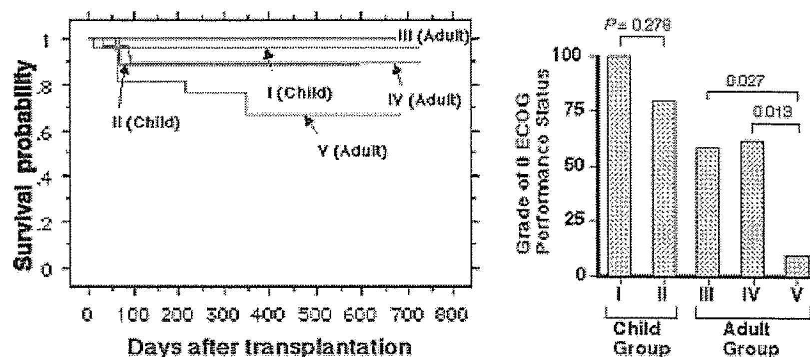

Figure 3. Kaplan-Meier curves showing recipient survival probability in the 5 (Groups I and II in child; Groups III, IV, and V in adult) groups (left). Percentage of grade 0 Eastern Cooperative Oncology Group performance status at 1 yr after LDLT (right).

TABLE 4. Comparison of Frequencies of Rejection and Infection in 5 Groups

Group	n	Rejection (%)	P*	Infection (%)	P*
Child					
I	24	58.3	—	54.2	—
II	9	66.7	1.000 (vs. group I)	44.4	0.708 (vs. group I)
Adult					
III	26	30.8	—	30.8	—
IV	30	23.3	0.561 (vs. group III)	46.7	0.279 (vs. group III)
V	23	30.4	1.000 (vs. group III)	69.6	0.010 (vs. group III)

*P-values are based on Fisher's exact test.

cytotoxic activity. In Groups IV and V, in contrast, $CD8^+$ T cells with very low proportions of naive T cells already had high cytotoxic activity prior to LDLT. The greater the $CD8^+$ CTL activity prior to LDLT, the smaller the capacity to generate CTLs for new invasion of bacteria and virus after LDLT (data not shown). Accordingly, the capacity to generate CTLs for infection after LDLT decreases progressively from Group I to Group V recipients, indicating progressive reduction in the latent ability to generate CTLs for clearance of new antigen. As a result, frequencies of postoperative complications are highest in Group V.

Current immunosuppressive induction protocols involve calcineurin inhibitors (cyclosporin, tacrolimus), corticosteroids, mono- and polyclonal antibodies, azathioprine, and mycophenolate mofetil. Use of steroids significantly increased the level of viremia in HCV-positive patients. Gane et al.[35] showed clearly that steroid pulse therapy is associated with a 4- to 100-fold increase in HCV-ribonucleic acid levels and subsequent development of acute hepatitis. In the present study, a Group IV recipient developed graft failure following repeated injection with steroid. On the other hand, in Groups IV and V, various immunosuppressors such as tacrolimus, cyclosporin, and others did not reduce preexisting CTL levels prior to transplantation. These levels had been reached during a lifetime of antigen exposure; there was also reduced thymopoiesis, characteristic of advancing age. In this regard, various conventional immunosuppressive agents remain limited in their ability to reduce preexisting CTLs. Drastic lymphocyte-depleting agents, such as rabbit anti-thymocyte globulin and anti-interleukin-2 antibodies, are able to induce cell regeneration by homeostasis-driven proliferation of T cells, and consequently provide the conditions involved in lymphocyte repopulation, favoring phenotypes with a low CTL activity with high naive T cells. Use of a preoperative shot pulse of antibody followed by low-dose immunosuppressive maintenance monotherapy may be reasonable compromise for a given transplant patient, based on preoperative high CTL activity.[36,37] However, we routinely perform anti-infective prophylaxis with less immunosuppression in Group V. It is very difficult to adjust the dose of immunosuppressive drugs specific for each recipient during infection.

Although this study had a short posttransplantation follow-up, the effect of the immunological status of T cells on the outcome has been definitively settled. Longer follow-up of larger cohorts is needed to decide whether the impairment of innate and adaptive immunoresponses by various dangerous factors after LDLTs has a significant adverse effect on long-term graft and patient survival.

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Beneficial Effects of Short-Term Lamivudine Treatment for *de novo* Hepatitis B Virus Reactivation After Liver Transplantation

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Clearance of hepatitis B surface antigen (HBsAg) by lamivudine is achieved in only a small proportion of patients with chronic hepatitis B virus (HBV) infection. We investigated the effect of lamivudine on *de novo* HBV reactivation after living-donor liver transplantation when the number of HBV was expected to be very small. Thirty-eight HBV-naive recipients who received liver grafts from antibodies to core antigen-positive donors receiving hepatitis B immunoglobulin (HBIG) were studied. HBsAg appeared in nine cases (23.7 %) despite receiving HBIG for 12–71 months (mean: 35.1 months) after transplantation. Lamivudine treatment was started in six recipients during the acute phase of HBV reactivation. Five of the six recipients achieved complete clearance of HBsAg in sera at a median of 4.6 months (ranging from 21 to 330 days) after lamivudine administration. Although lamivudine was stopped in four cases, all remained negative for HBsAg. Our findings suggested that short-term lamivudine treatment during acute phase of HBV reactivation could achieve complete clearance of HBsAg in a significant number of liver transplant recipients.

Key words: Anti-HBc, hepatitis B, hepatitis B immunoglobulin, lamivudine, liver transplantation

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Introduction

There is growing recognition that the majority of healthy individuals who are negative for hepatitis B surface antigen (HBsAg) but positive for antibodies to core antigen (anti-HBc), who had once been assumed to denote previous exposure to hepatitis B virus (HBV), have persistent viral infection of the liver tissues (1,2). Recently, we demonstrated that latent HBV infection is accompanied by ongoing viral replication in the livers, but not in the sera, of healthy anti-HBc-positive liver transplant donors (3). In support of the concept of occult HBV infection, HBV was transmitted from anti-HBc-positive donors to HBV-naive recipients at a high frequency via liver grafts in living-donor liver transplantation (LDLT) and orthotopic liver transplantation (4–8). Because of the persistent shortage of organs and the increasing number of patients awaiting transplantation, the use of liver grafts from anti-HBc-positive donors cannot be avoided, especially in areas where the prevalence of HBV is high (4). Thus, adequate prophylaxis against HBV is required to prevent viral reactivation in HBV-naive recipients after liver transplantation.

To date, strategies to prevent viral breakthroughs in the recipients of anti-HBc-positive livers have been empirical, and hepatitis B immunoglobulin (HBIG) has been widely used as the standard prophylaxis after liver transplantation (9). Several reports, including ours, suggested that HBIG prophylaxis was effective for preventing HBV exacerbation in recipients who received hepatic allografts from anti-HBc-positive donors (4,10). However, long-term passive immunization with HBIG is associated with problems, such as high cost, limited availability, and selection of viral strains containing mutations in the surface gene of HBV-DNA (9). Moreover, the difficulty of maintaining serum antibodies to HBsAg (anti-HBs) titer and the poor compliance of HBIG could result in viral reactivation after liver transplantation (7).

The aim of this study was to evaluate the efficacy of short-term administration of lamivudine for the treatment of *de novo* HBV exacerbation in transplant recipients with anti-HBc-positive donors.

Patients and Methods

Patients

From July 1995 to July 2004, 902 patients underwent LDLT at Kyoto University Hospital. Before operations, serological evaluation for HBV markers, including HBsAg, anti-HBs, anti-HBc, hepatitis B e antigen (HBeAg), and antibodies to HBeAg (anti-HBe), was carried out using commercial enzyme immunoassay kits (Dainabot, Tokyo, Japan). HBV-DNA was analyzed using a commercial polymerase chain reaction (PCR) assay (Amplicor HBV Monitor, Roche, Branchburg, NJ, USA). Among 902 donors, 121 (13.4%) were positive for anti-HBc in the absence of HBsAg. Of these, all recipients fulfilling both of the following criteria were included: (a) none of the HBV-related

serological markers positive before transplantation; (b) post-operative survival and well-tolerated prophylaxis with HBIG for longer than 6 months after LDLT. Accordingly, 55 recipients were excluded because they were positive for HBV-serological markers before LDLT. Twenty-six patients were also excluded from the study because of their short duration of survival, and all of these patients died from causes not related to the HBV reactivation. Two patients refused to receive HBIG for financial reasons, and were lost to follow-up. A total of 38 recipients were considered eligible for this study. These HBV-naive recipients with anti-HBc-positive donors underwent LDLT for the following liver diseases: biliary atresia (n = 21), hepatitis C virus (HCV)-related chronic liver disease (n = 3), primary biliary cirrhosis (n = 3), primary sclerosing cholangitis (n = 2), chronic rejection (n = 2), post-LDLT graft failure (n = 2) and others (n = 5). The male/female ratio was 1/1 and the age range was 0–58 years (mean age: 15.9 years, age ≤18 years: n = 28, age >18 years: n = 10). HBV reactivation after LDLT was diagnosed by confirming the appearance of HBsAg in sera of the recipients. Liver tissue and serum samples of all anti-HBc-positive donors were obtained at the time of operation and subjected to analysis for HBV-DNA. All subjects provided written informed consent, and the study was conducted in accordance with the principles of the Declaration of Helsinki.

Prophylaxis with HBIG and immunosuppressive protocol

HBIG monotherapy was given to all the recipients with grafts from anti-HBc donors, as reported previously (4). The first dose of HBIG at 200 IU/kg body mass was administered during the anhepatic phase of LDLT, and the same dose was given every day during the first 6 post-operative days. Subsequently, HBV-serological markers were examined at monthly intervals after the transplant operation and 1000 IU of HBIG was periodically administered to maintain serum anti-HBs titers at more than 200 IU/L throughout the follow-up period.

Immunosuppressive therapy for all recipients consisted of tacrolimus and low-dose steroids. Target trough levels of tacrolimus in whole blood were 10 to 15 ng/mL in the first week, and then 5 to 10 ng/mL during the first month after transplantation. Methylprednisolone (10 mg/kg) was administered intravenously (IV) during the anhepatic phase of surgery, followed by 2 mg/kg administered IV for the first 3 days, then tapered to 1 mg/kg for 3 days and converted to 0.3 mg/kg/day of prednisone, which was decreased gradually and discontinued between 3 and 6 months.

PCR amplification of HBV-DNA and sequencing of the surface gene

Preparation of DNA samples and detection of HBV genomes by nested PCR have been described previously (3). The nucleotide sequence spanning the S region was amplified by PCR using specific primer sets, followed by subcloning of PCR products using a pGEM-T Easy Vector System I (Promega, Madison, WI, USA). A total of 15 clones derived from each serum specimen were subjected to sequencing analyses (3).

Results

HBV reactivation despite HBIG prophylaxis

Post-operative HBIG prophylaxis was given to 38 HBV-naive recipients with grafts from anti-HBc-positive donors. Among them, 29 showed no evidence of HBV recurrence during the follow-up period (mean: 41.1 months, range: 10 months to 9.5 years). Unfortunately, in 9 of 38 cases (23.7 %), anti-HBs titer decreased concurrently with the appearance of HBsAg in the serum despite HBIG prophylaxis after LDLT. Table 1 shows the serological characteristics of the donors and pre-transplant status of the recipients who suffered from HBV reactivation despite HBIG administration. Baseline characteristics including age, gender and HBV-related serology were similar between these nine recipients and the remaining recipients without HBV recurrence (data not shown). Consistent with our previous analyses, 31 of the 38 donors with anti-HBc (81.6%) were positive for HBV-DNA in the liver specimens, indicating a high frequency of latent HBV infection in the livers of anti-HBc-positive individuals (3). In contrast, HBV-DNA was negative in the sera of all anti-HBc-positive donors. All allografts of nine recipients with HBsAg appearance were positive for HBV-DNA by PCR analyses, suggesting that the *de novo* HBV reactivation originated from the liver graft with latent HBV infection after LDLT.

To define the factors associated with HBV reactivation in these nine recipients, variables related to the donors and recipients, transplant procedures, and HBIG prophylaxis were analyzed. The indications for LDLT in these recipients with HBV reactivation were biliary atresia (n = 7), Wilson's disease (n = 1) and primary sclerosing cholangitis (n = 1). The mean period between LDLT and *de novo* HBsAg appearance was 35.1 months (range: 12–71 months; Table 2).

Table 1: HBV-serological status of recipients with HBV reactivation post-LDLT and of their corresponding donors with anti-HBc

Recipient				Donor			
Case #	Age/Sex	Indication for LDLT	HBsAg/ Anti-HBs	Anti-HBc	HBsAg/ Anti-HBs	HBeAg/ Anti-HBe	HBV-DNA in liver graft
1	13/F	BA	–/–	+	–/+	–/+	+
2	3/M	BA	–/–	+	–/+	–/–	+
3	9/M	BA	–/–	+	–/+	–/+	+
4	22/F	BA	–/–	+	–/+	–/–	+
5	0/M	BA	–/–	+	–/+	–/+	+
6	16/F	BA	–/–	+	–/+	–/+	+
7	16/M	Wilson	–/–	+	–/–	–/+	+
8	23/F	BA	–/–	+	–/–	–/–	+
9	25/M	PSC	–/–	+	–/–	–/+	+

LDLT = living-donor liver transplantation; HBsAg = hepatitis B surface antigen; anti-HBs = antibody to HBsAg; anti-HBc = antibody to hepatitis B core antigen; HBeAg = hepatitis B e antigen; anti-HBe = antibody to HBeAg; BA = biliary atresia; PSC = primary sclerosing cholangitis.

Table 2: Clinical features of recipients with HBV reactivation

Case #	Anti-HBs titer[1] (mIU/mL)	Duration until HBV reactivation[2] (months)	Clinical features at the time of HBV reactivation		Possible reasons for HBV reactivation
			ALT (IU/L)	Histology	
1	N.D.	21	251	N.D.	Noncompliance
2	N.D.	32	190	CAH	Noncompliance
3	N.D.	16	13	N.D.	Noncompliance
4	23.8	71	699	N.D.	Immunosuppression
5	140.6	15	24	N.D.	Escape mutant
6	117	30	153	CAH	Escape mutant
7	11.7	12	1409	CAH	Unknown
8	N.D.	61	25	CAH	Unknown
9	34.7	58	65	CAH	Unknown

anti-HBs = antibody to HBsAg; ALT = alanine aminotransferase; N.D. = not determined; CAH = chronic active hepatitis; noncompliance = noncompliance of HBIG; escape mutant = emergence of surface escape mutant.
[1]Anti-HBs titer before HBsAg appearance.
[2]Period between liver transplantation and HBsAg appearance.

A liver biopsy was performed on five of the nine patients at the time of the *de novo* HBV recurrence, and all exhibited evidence of chronic active hepatitis accompanied by mild inflammatory activity and mild fibrosis. HBV appearance was attributed to the decrease in serum anti-HBs titer despite HBIG prophylaxis in four of nine recipients. Among them, three recipients (cases #1, #2 and #3) were considered to have suffered from HBV recurrence because of non-compliance of HBIG. Although post-operative HBIG prophylaxis was given to these three patients, they had a transient cessation of HBIG treatment for personal reasons 12, 7 and 11 months after LDLT. They experienced a decrease in anti-HBs titer and, consequently, HBsAg became detectable in the sera after cessation of HBIG treatment. The mean period between the cessation of HBIG treatment and the emergence of HBsAg in their sera was 12.3 months (range, 3–26 months). An immunosuppressive condition was presumed to be associated with viral activation, with consequent decreases of anti-HBs in one case (case #4). Recipient #4 showed an anti-HBs titer of less than 23.8 IU/L 2 weeks after the 2000 IU-HBIG infusion, followed by the appearance of HBsAg. Continuous medication with prednisolone for the treatment of chronic rejection suggested the underlying possible immunocompromised condition in this case.

In the remaining five cases (cases #5, #6, #7, #8 and #9), HBsAg eventually became positive despite the continuous treatment with periodical HBIG prophylaxis. HBV clones comprising mutations in the S gene have been reported in OLT recipients who developed recurrent hepatitis B despite HBIG prophylaxis (11). To ascertain whether the HBIG failure in these cases was associated with changes in antigenicity of the S protein, the S gene sequence of HBV-DNA was determined in the HBV strain of two patients whose sera at the acute phase of HBV exacerbation were available for further analyses (cases #5 and #6). The sequence analyses of the two cases revealed that the detected HBV clone contained several mutations, including G- to -A substitutions at nucleotide 586 (subtype adr) and 587 (subtype adw) within the 'a' determinant region on the HBsAg-encoding gene, suggesting that the cloned HBV variants might be responsible for HBV recurrence despite HBIG administration in these two cases. Unfortunately, we could not determine the factors that were related to HBV reactivation in cases #7, #8 and #9.

Short-term lamivudine treatment

Among recipients with *de novo* HBV recurrence, six (cases #1, #2, #4, #6, #7 and #9) of nine cases had elevated levels of serum alanine aminotransferase, suggesting that recurrent active hepatitis was present. Moreover, of these six cases, all four cases that were examined exhibited histological evidence of inflammation with lymphocytes infiltration around the portal area at the time of *de novo* HBV reactivation (Table 2). Lamivudine (100 mg) was given in six cases to suppress the viral activity (Figure 1). Of them, five patients started the treatment immediately after HBsAg appearance (average: 27 days; range: 1 day to 2 months). One patient (case #1) did not take lamivudine at the time of HBsAg appearance because of personal reasons, but finally received lamivudine therapy 23 months after HBV reactivation.

After the administration of lamivudine, HBsAg decreased in the sera of five out of the six recipients, and in all these five cases (cases #1, #2, #4, #7 and #9), HBsAg disappeared from the sera at a median of 4.6 months (range: 21–330 days) after the beginning of lamivudine treatment. Suppression of HBsAg by lamivudine treatment was invariably associated with a decline in serum transaminase levels in these five cases. After confirming the stable seroconversion to anti-HBs-positive status, lamivudine treatment was stopped in four of the five recipients after 60 months, 10 months, 1.5 months and 4 months (cases #1, #2, #7 and #9). The remaining individual (case #4) is currently receiving lamivudine treatment because she started treatment only 4 months ago; her most recent blood test

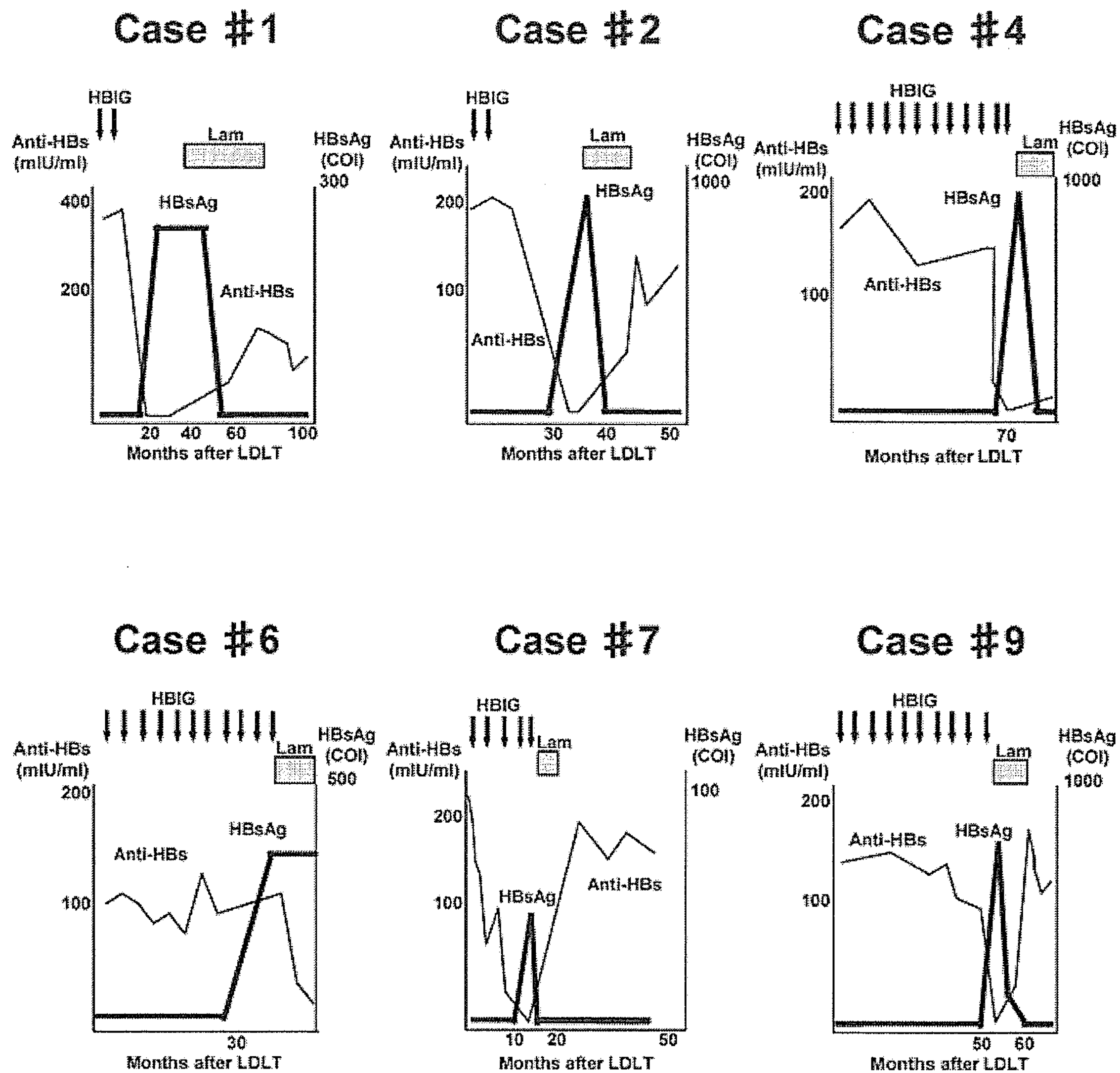

Figure 1: Clinical course of the six liver transplant recipients with HBV reactivation who received lamivudine treatment. The bold line represents HBsAg and the fine line represents anti-HBs. The treatment with HBIG is shown as arrows and treatment with lamivudine as shaded boxes. HBIG = hepatitis B immunoglobulin; Lam = lamivudine.

was negative for HBsAg. The presence of circulating exogenous anti-HBs derived from HBIG complicates detection of the endogenous anti-HBs, which reflect the development of anti-HBV immunity in recipients. Thus, we also gradually withdrew the HBIG prophylaxis after the administration of lamivudine, and confirmed the sustained positivity of anti-HBs without HBIG treatment in two recipients (cases #2 and #7). Since then, they consistently showed evidence of immunity against HBV with endogenous anti-HBs titers greater than 100 IU/L without any prophylaxis. Consequently, HBIG was not given to these two recipients, even after termination of lamivudine treatment. Serum HBsAg of these two recipients remained negative during the follow-up periods of 14 and 35 months after the withdrawal of HBIG and lamivudine. Recipient #7 died of progressive deterioration of liver function 35 months after the complete loss of HBsAg in sera, and the histological autopsy findings were consistent with chronic rejection in the liver graft. The remaining recipient (case #6) is currently being treated with lamivudine monotherapy. None of the recipients developed tyrosine-methionine-aspartate-aspartate (YMDD) mutants during the course of lamivudine therapy (data not shown).

Three patients (recipients #3, #5 and #8) did not receive lamivudine treatment after HBsAg appearance because the alanine aminotransferase levels of these patients showed no evidence of active hepatitis.

Discussion

In this study, we demonstrated that short-term lamivudine therapy for LDLT recipients with *de novo* HBV reactivation