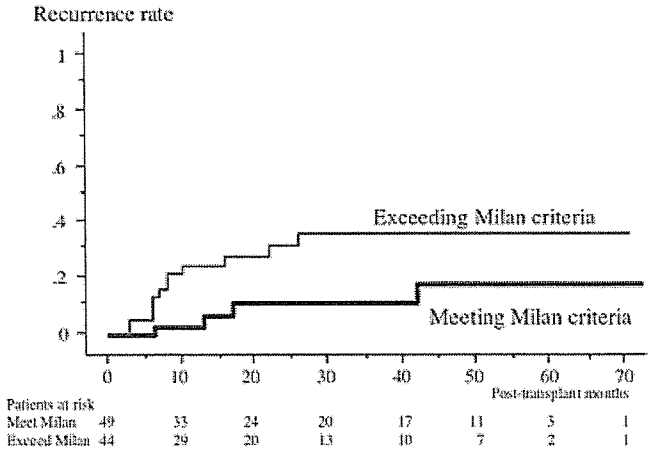

Figure 2. Cumulative recurrence rate according to MC. Cumulative HCC recurrence rate after LDLT was significantly higher for patients who exceeded MC (n = 44, 35%) than for patients who met MC (n = 49, 15%; *P* = 0.0190).

Pathological Findings and Recurrence

Correspondence of preoperative MC with the results of pathological analysis was evaluated in postoperative examinations of explanted livers. Completely ablated foci (100% necrosis) following pretransplant treatments were not counted as tumor. On pathological examination, 39 patients met so-called "pathological MC" and 54 did not. Accordingly, preoperative imaging underestimated the diagnosis in 13 patients (14%) and overestimated in 3 patients (3%). Of the 13 underestimated patients, HCC recurred in 4 patients. Univariate analysis of pathological findings (Table 3) revealed that tumor diameter >5 cm, tumor number ≥4, tumor grade indicating poor differentiation, and positive microvascular invasion were all significantly associated with postoperative recurrence. Using Cox's multivariate analysis (Table 4), pathological tumor number and poor differentiation were identified as independent risk factors for recurrence.

Effects of Previous Treatment Before LDLT

Patients were divided into 3 groups based on history of conventional treatment for HCC before LDLT: patients who primarily received LDLT without previous treatment (Group 1, n = 20); patients with 1-2 treatments (Group 2, n = 30); and patients with ≥3 treatments (Group 3, n = 43). Median period between first diagnosis of HCC and LDLT was 3 months in Group 1 (range, 1-15 months), 14 months in Group 2 (range, 2-70 months), and 36 months in Group 3 (range, 4-168 months). Preoperative liver function was most deteriorated in Group 1. Mean (± SD) preoperative MELD score was significantly higher in Group 1 (21 ± 8) than in Groups 2 (16 ± 7, $P = 0.0414$) or 3 (12± 6, $P = 0.002$). Conversely, the proportion of patients who exceeded MC before LDLT was significantly higher in Group 3 (63%) than in Groups 1 (35%) or 2 (33%, $P = 0.020$).

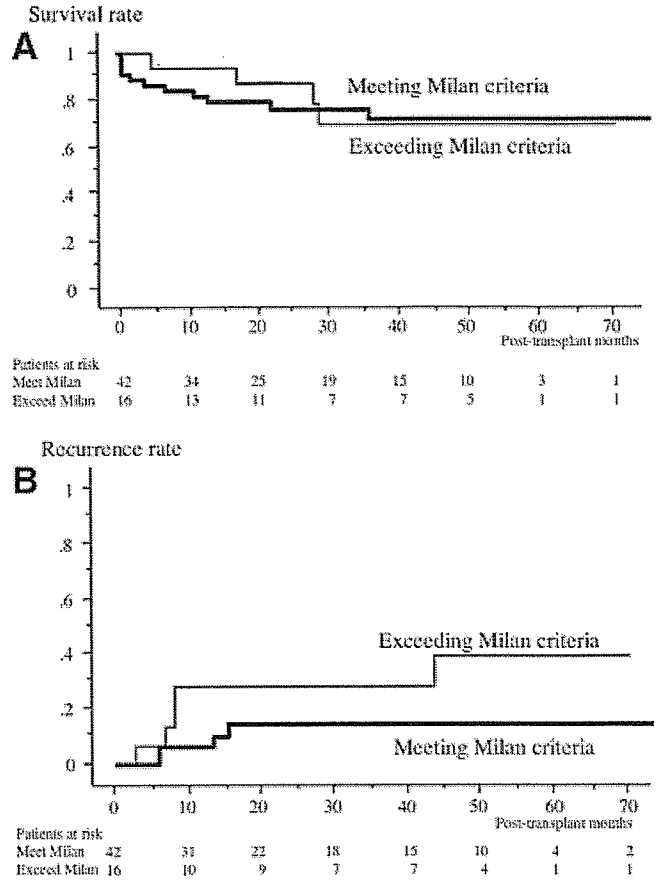

Figure 3. Survival and recurrence rates according to MC at first diagnosis of HCC.For the 58 patients who had a history of previous nontransplant treatment for HCC and for whom records of tumor stage prior to any treatment were available, patient survival and HCC recurrence rates after LDLT were determined according to MC based on imaging at first diagnosis of HCC. (A) Survival rates were similar for patients who had met MC (n = 42) and those who had not met MC (n = 16) (71% vs. 69% at 4 years after LDLT, respectively; *P* = 0.8259). (B) Recurrence rates tended to be higher in patients who had exceeded MC than in patients who had met these criteria, although differences were not significant (37% vs. 13%, respectively; *P* = 0.0699).

Based on postoperative examinations of explanted livers, microscopic venous invasion was significantly more frequent in Group 3 (51%) than in Groups 1 (21%) or 2 (33%, $P = 0.0382$). Postoperatively, HCC recurrence occurred in 3 patients in Group 1, 3 patients in Group 2, and 10 patients in Group 3. Survival rates at 4 years tended to be better for Group 2 (80%) than for Groups 1 (52%) and 3 (58%), although these differences were not significant ($P = 0.0651$ and $P = 0.1042$, respectively; Fig. 4A). Recurrence rates at 4 years were 23% for Group 1, 9% for Group 2, and 37% for Group 3. Rates were significantly lower for Group 2 than for Group 3 ($P = 0.0411$; Fig. 4B).

DISCUSSION

The present study showed that advanced stage of tumors in terms of tumor size, number, and α-fetoprotein

TABLE 3. Univariate Analysis of Pathological Tumor Characteristics and Recurrence Rate

Variables		n	4-Year Recurrence Rate	P
Tumor size	>5 cm	14	55%	0.0031
	≤5 cm	79	19%	
Tumor number	≤3	48	5%	0.0004
	≥4	45	49%	
Differentiation	Well	14	0%	
	Moderate	59	23%	0.0001*
	Poor	20	60%	
Microscopic venous invasion	Positive	37	42%	0.0026
	Negative	56	14%	

*Comparison between "Moderate" and "Poor."

TABLE 4. Multivariate Analysis of Pathological Tumor Factors and Recurrence

Variables		Risk Ratio	95% Confidence Interval	P
Tumor number	≤3	1		
	≥4	7.917	1.799-20.000	0.002
Differentiation	Well or Moderate	1		
	Poor	5.618	1.712-18.519	0.003

levels on preoperative evaluation is associated with increased postoperative recurrence. These results corroborate the findings of previous studies on deceased donor liver transplantation.[19-22] In a study by Gondolesi et al.[23] of 36 patients with HCC treated using LDLT, tumor size >5 cm showed no significant effect on recurrence. Similarly, in our preliminary report on 56 patients,[12] recurrence rate did not differ between patients within and beyond MC. However, as patient numbers increased, the present study demonstrated that patients with tumors >5 cm in diameter experienced significantly higher rates of recurrence and mortality. In addition, patients who exceeded MC displayed significantly higher risk of recurrence.

Of note was the finding that patient survival rates were similar for patients who met MC and for patients who did not (Fig. 1). This discrepancy may be due to a short follow-up period, but it can also be explained by HCC-unrelated deaths due to postoperative complications occurring within a few months after LDLT. Infectious complications such as sepsis, pneumonia, and peritonitis were the most common causes of early mortality. As a rule, patients with early HCC were referred to our hospital for LDLT due to advanced liver cirrhosis. Mortality rates within 3 months were 18% for those within MC and 9% for those beyond MC, which may be associated with poorer preoperative condition for the former group as reflected by higher MELD score (18 ± 8 vs. 13 ± 6, respectively; $P = 0.002$). Likewise, most patients who primarily received LDLT without previous treatment (Group 1, Fig. 4) were characterized by higher MELD scores and less advanced tumors. Survival rates for these patients were compromised by HCC-unrelated death due to perioperative complications.

In the present study, 78 patients had received nontransplant treatments for HCC prior to LDLT. For these patients, tumor staging was determined by counting only viable and enhancing nodules on pretransplant computed tomography. Pretransplant tumor staging thus differed from that at first diagnosis of HCC. In the 58 patients for whom records of tumor stage prior to any treatment were available, 6 patients with tumors originally beyond MC had been down-staged with treatment and were assigned to the within-MC group before LDLT. Conversely, 18 patients were up-staged before LDLT. In an attempt to analyze outcomes according to primary tumor staging, survival and recurrence curves were determined for patients meeting and patients exceeding MC based on imaging at first diagnosis of HCC for the 58 patients (Fig 3). While patient survival rates did not differ significantly, recurrence rate tended to be higher in patients who had originally exceeded MC than in patients who had met MC. However, these differences were not significant, probably due to small number of patients involved.

Another controversy remains regarding indications for patients with early HCC. On the one hand, use of LDLT has been proposed for patients with early HCC accompanied by early-stage cirrhosis (Child-Turcotte-Pugh A), as a reasonable survival rate should be expected in weighing the risk of live donation.[24] Conversely, risking the life of a live donor for a patient who has alternative options of hepatic resection or other curative treatments with comparable long-term survival is unlikely to be ethically acceptable.[25] Patient

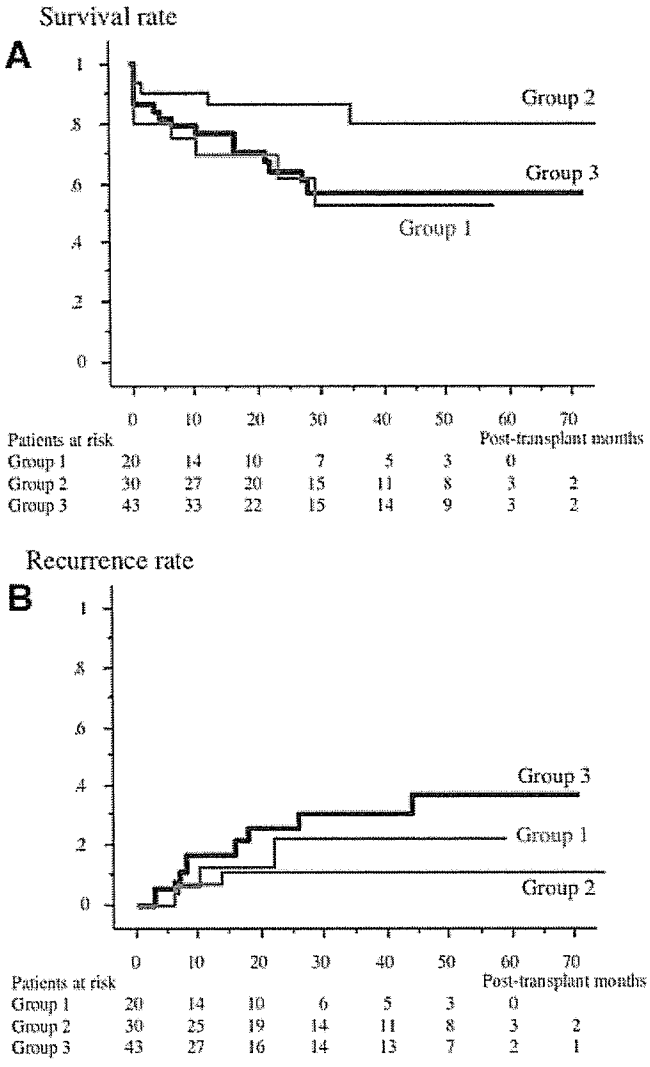

Figure 4. Survival and recurrence rates according to history of previous HCC treatment. According to history of conventional treatment for HCC before LDLT, patients were divided into 3 groups: Group 1, patients without previous treatment (n = 20); Group 2, patients with 1-2 treatments (n = 30); and Group 3, patients with ≥3 treatments (n = 43). (A) Survival rates at 4 years tended to be better for Group 2 (80%) than for Groups 1 (52%) and 3 (58%), although differences were not significant (*P* = 0.0651 and *P* = 0.1042, respectively). (B) Recurrence rate at 4 years was 23% for Group 1, 9% for Group 2, and 37% for Group 3. Recurrence rate was significantly lower for Group 2 than for Group 3 (*P* = 0.0411).

selection in our hospital is based on the latter policy: Patients with HCCs considered unsuitable for resection or local ablation therapies have been included in our program. In preoperative evaluations, a system for precise prognostic staging is essential for comparing outcomes between groups undertaking different therapeutic trials. Kudo et al.[14] recently reported that JIS score offers a better system of prognostic staging for HCC than previous systems, in terms of both stratification ability and prognostic predictive power. In an analysis of 4,525 patients with HCC who received various conventional therapies, including hepatic resection, percutaneous ablation therapies and transarterial chemoembolization, patient survival was clearly stratified according to JIS score. The 5-year survival rates for patients were 73% for JIS 0 (n = 552), 52% for JIS 1 (n = 1,399), 33% for JIS 2 (n = 1,471), 13% for JIS 3 (n = 757) and 2% for JIS 4 (n = 244).[14] This score system has not yet been validated outside Japan,[26] but it was applied to our series of LDLT recipients. As a result, both 4- and 5-year survival rates were 58%, even in JIS 4 patients. Although these figures should be carefully compared, LDLT may result in improved prognosis for patients with a JIS score ≥2.

Conversely, for patients with early HCC and preserved liver function (that is, JIS score of 0 or 1), hepatic resection or ablation therapies would represent the treatments of choice. However, high rates of recurrence occur even after curative treatment,[27] and the role of secondary or salvage transplantation has recently been discussed.[24,25,28,29] Although prior hepatic resection may complicate the operative transplant procedure and increase the risk of postoperative complications,[24] postoperative survival in the 11 patients who received hepatic resection before LDLT was comparable to survival in the other 82 patients in this series (Table 1). On the other hand, transplantability at the time of recurrence is supposedly limited due to advanced tumor extension.[24] Even for patients who are considered eligible for salvage LDLT, the present study revealed significantly higher recurrence rates for patients with a history of ≥3 treatments for HCC before LDLT (Group 3) than for patients with only 1-2 treatments (Group 2, Fig. 4B). This result implies that repeated nontransplant treatments for recurrent HCC may increase the risk of microscopic vascular invasion and impair the survival advantages conferred by LDLT. For patients who develop HCC recurrence after conventional therapies, feasibility, optimal timing, and efficacy of LDLT as a second-line treatment should be determined in further studies.

In conclusion, although efforts to decrease early mortality due to surgical complications are essential to improve outcomes for LDLT, this technique may constitute an optional treatment with a chance of cure for patients displaying otherwise uncontrolled HCC. Patients who develop recurrent HCC should be referred for LDLT before repeated nontransplant therapies, if a living donor is available.

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Identification of novel defective HCV clones in liver transplant recipients with recurrent HCV infection

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SUMMARY. Patients with recurrent hepatitis C after liver transplantation usually have a high viral load and are generally resistant to interferon (IFN)-α2b plus ribavirin (RBV) therapy. However, it remains unclear whether pretreatment viral titre determines the effectiveness of combination therapy, especially in patients with a high viral load. The aim of this study was to identify the viral factors associated with a sustained virological response (SVR) to antiviral therapy in patients with recurrent hepatitis C after living-donor liver transplantation. Twenty-three patients with recurrent hepatitis C received combination therapy of IFN-α2b plus RBV. SVR was achieved in 7 of the 23 patients (30.4%). Predictive factors for SVR included a 2 $\log_{10}$ decline in Hepatitis C virus (HCV) RNA at 2 weeks after the start of therapy and disappearance of HCV RNA at 4 or 24 weeks after the start of therapy. As the pretreatment high viral load showed no association with SVR, we asked whether other viral factor was associated with the response to the combination therapy in transplant recipients. We found the several novel defective HCV clones in 4 of 12 recipients' sera. All defective HCV clones had deletions in the envelope region. Interestingly, no patients with defective clones showed a prompt decrease in HCV RNA after the start of IFN-α2b plus RBV therapy. Thus, early decline in serum HCV RNA after treatment was closely associated with SVR. The circulating defective HCV clones are present and might be associated with the response to the combination therapy in patients with recurrent hepatitis after liver transplantation.

Keywords: hepatitis C virus genome, interferon, liver transplantation, ribavirin, sustained virological response.

INTRODUCTION

Hepatitis C virus (HCV) infection is one of the leading causes of end-stage liver disease; thus, HCV-related chronic liver disease is a common indicator for liver transplantation. However, recurrent hepatitis in the liver allograft develops in most recipients [1]. Unlike immunocompetent individuals, HCV recurrence in patients who receive a liver transplant results in severe liver damage at an accelerated rate, leading to recurrent cirrhosis within 5 years [2,3]. Moreover, several investigators have shown that HCV recurrence is more severe in living-donor liver transplantation (LDLT) than in orthotopic liver transplantation (ORLT) [4,5]. LDLT provides the only access to donor organs in countries like Japan, where cadaveric donors are not available. Therefore, establishment of highly effective treatment strategies for recurrent hepatitis C after liver transplantation is strongly required.

Hepatitis C in transplant recipients differs from that in immunocompetent individuals in several aspects [6]. Most recipients have extremely high viral loads as a result of their immunosuppressive conditions [6]. HCV baseline viral load is an independent factor associated with poor response to interferon (IFN)-α2b plus ribavirin (RBV) combination therapy [7,8]. Thus, liver transplant patients with recurrent hepatitis C are generally resistant to antiviral treatment. In fact, sustained virological response (SVR) in ORLT recipients by IFN-α2b plus RBV combination therapy has only been observed in up to 30% of patients [9,10]. Thus, resistance to

Abbreviations: IFN, interferon; RBV, ribavirin; SVR, sustained virological response; LDLT, living-donor liver transplantation; HCV, hepatitis C virus; ORLT, orthotopic liver transplantation; PMN, polymorphonuclear; HCC, hepatocellular carcinoma; HLA, human leukocyte antigen; MU, million units; RT-PCR, reverse transcriptase-polymerase chain reaction; HBV, hepatitis B virus; 5′-UTR, 5′-untranslated region; NS, nonstructural protein; E1, envelope glycoprotein-1; E2, envelope glycoprotein-2.

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IFN-α2b plus RBV combination therapy in recipients with HCV recurrence is a challenging obstacle at present. Therefore, the aim of this study was to determine viral factors other than genotype 1b and high viral load that influence responsiveness to IFN-α2b plus RBV combination therapy in patients with recurrent hepatitis C after LDLT.

MATERIALS AND METHODS

Patients

Between March 1999 and December 2004, 100 patients underwent LDLT at Kyoto University Hospital as a result of end-stage liver disease caused by HCV infection. Recurrence of chronic active hepatitis C was histologically confirmed on a biopsy sample in 49 patients. Indicators for IFN-α2b plus RBV combination therapy in these patients included high serum values of alanine aminotransferase (above upper limits of normal >27 IU/L), with haemoglobin levels >8 g/dL, total polymorphonuclear (PMN) counts >1500/mm^3, platelet counts >50 000/mm^3, normal renal function (serum creatinine <1.5 mg/dL and/or creatinine clearance >50 mg/mL) and no hepatocellular carcinoma (HCC) recurrence. Of 49 patients, 30 received treatment with IFN-α2b plus RBV combination therapy (Schering-Plough, Kenilworth, NJ, USA). Among them, 23 patients, who were sequentially monitored at the same hospital throughout the treatment period and 24 weeks after cessation of treatment, were enrolled for further analyses. At the time of entry, liver biopsy specimens were assessed by an experienced hepatopathologist using the METAVIR score; the fibrosis stage was defined as F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis) and F4 (cirrhosis) [11]. The HCV genotype was determined using a commercially available reverse transcriptase-polymerase chain reaction (RT-PCR) assay that can distinguish genotypes 1a, 1b, 2a, 2b, 3a and 3b of HCV (Monitor HCV Coregenotyping; SRL, Tokyo, Japan). Serum HCV RNA was measured before, at 2, 4, 12, 24 and 48 weeks during therapy and every 4 weeks after the end of therapy, using the Roche Amplicor HCV 2.0 assay (Roche Diagnostics, Branchburg, NJ, USA). Serum samples were diluted and retested by the same assay, when serum HCV RNA load was over 850 kIU/mL. All patients received tacrolimus-based immunosuppression [12]. The ethics committee of Kyoto University approved the studies, and informed consent for participation in the study was obtained from all patients.

Typing of human leukocyte antigen class

We performed human leukocyte antigen (HLA) typing by PCR amplification with MicroSSP HLA DNA typing trays (One Lambda, Canoga Park, CA, USA) according to the manufacturer's instructions in patients. The amplified DNA was electrophoresed and visualized on ethidium-bromide-stained 2% agarose gels. HLA analysis included 17 HLA-A, 19 HLA-B and 16 HLA-DR alleles.

Study design

Patients were treated with 6 million units (MU) or 3 MU of recombinant IFN-α2b three times a week for 48 weeks (3 MU IFN-α2b was used when platelet counts were <100 000/mm^3 or PMN counts were <2000/mm^3 before therapy) plus oral RBV for the first 24 weeks. RBV dosage was based on the weight of each patient: patients less than 60 kg were administered 600 mg, and patients greater than 60 kg received 800 mg. IFN dosage was reduced to 3 MU if platelet counts dropped to <100 000/mm^3 or if PMN counts dropped to <2000/mm^3 during therapy. When haemoglobin levels before treatment were less than 10 g/dL, RBV dosage was reduced to 400 mg in patients weighing less than 60 kg and to 600 mg in patients weighing more than 60 kg. The combination therapy was discontinued when haemoglobin levels, total PMN counts or platelet counts were observed to be less than the eligible levels mentioned above during therapy. SVR was defined as no detectable HCV RNA by qualitative assay at least 24 weeks after cessation of therapy.

Detection of defective HCV RNA

Serum samples for the detection of defective HCV RNA were taken before the start of IFN-α2b and RBV administration. Total RNA was extracted from 250 μL of serum using Sepasol® RNA II super (Nacalai Tesque, Kyoto, Japan), according to the manufacturer's protocol. The extracted RNA was resuspended in 10 μL of RNase-free water and 5 μL of each RNA sample was used in each reaction. RT-PCR was performed using the One Step RNA PCR Kit (Takara, Tokyo, Japan), as described earlier [13]. The RT-PCR primers, which were designed from the reference sequence of HCV genotype 1b (GenBank Accession No. #D90208), were shown in Table 1. All PCR products were analysed by electrophoresis in 1.2% agarose gels stained with ethidium bromide. Subcloning of purified DNA was performed using the pGEM-T easy vector (Promega, Madison, WI, USA) [14]. At least 30 colonies on each plate were picked using white/blue colony selection, and plasmid DNA was extracted and then purified by ethanol precipitation. Each purified DNA sample was sequenced at least three times using an ABI Prism Big Dye Terminator Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) [14]. To determine the defects in the HCV genome, the sequence of each sample was compared with the registered HCV genome sequence, as described above. The serum of a healthy HCV-RNA-negative volunteer was used as a negative control. The full RNA genome of HCV was synthesized *in vitro* from the pM1LE plasmid (inserted genotype 1b sequence of HCV full genome) using a MEGAscript T7 Kit (Ambion, Austin, TX, USA) and used as a positive control [15].

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

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.

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

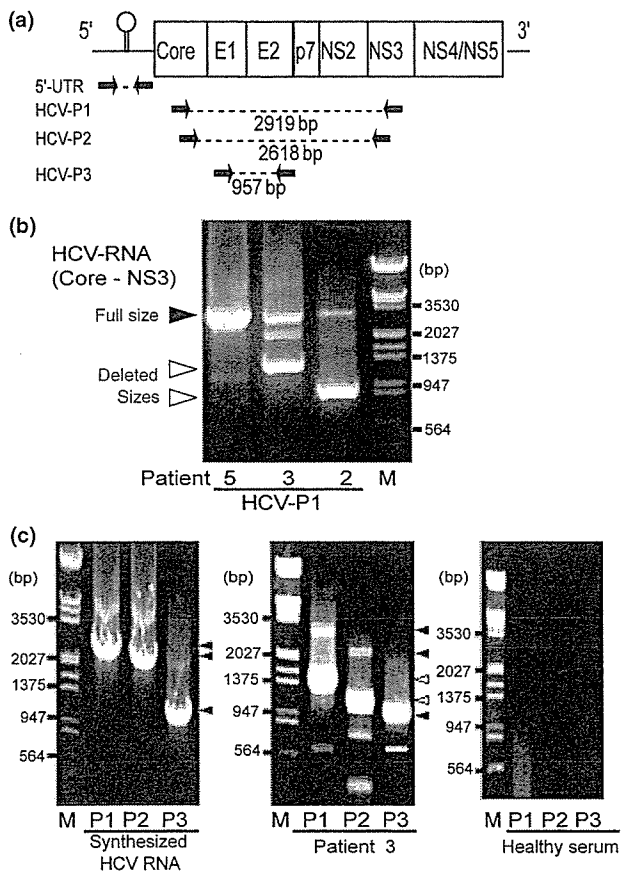
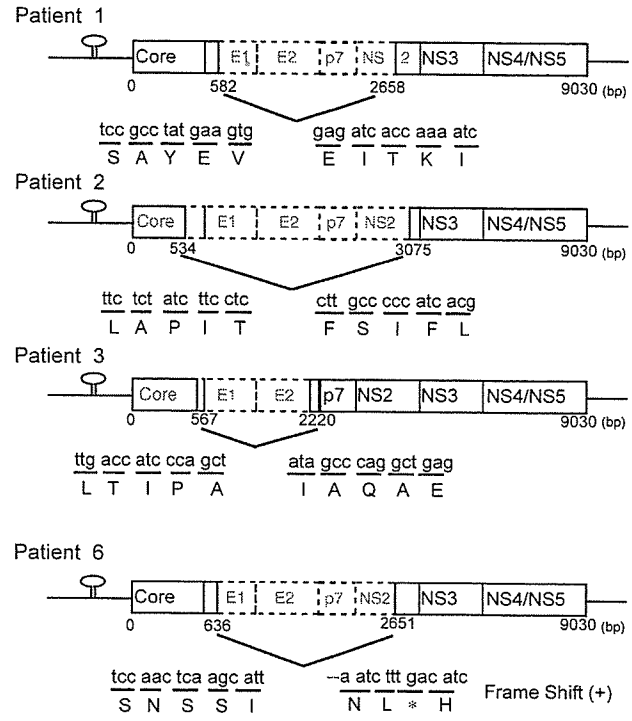


Fig. 1 Defective HCV clones detectable in the sera of highly viraemic patients with recurrent hepatitis C after LDLT. (a) Schematic presentation of the HCV genome and the primer sets used in this study. The sense and antisense primers specific for that part of the HCV sequence spanning 5′-UTR and NS3 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.

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.

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

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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LIVER TRANSPLANTATION 12:792-800, 2006

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

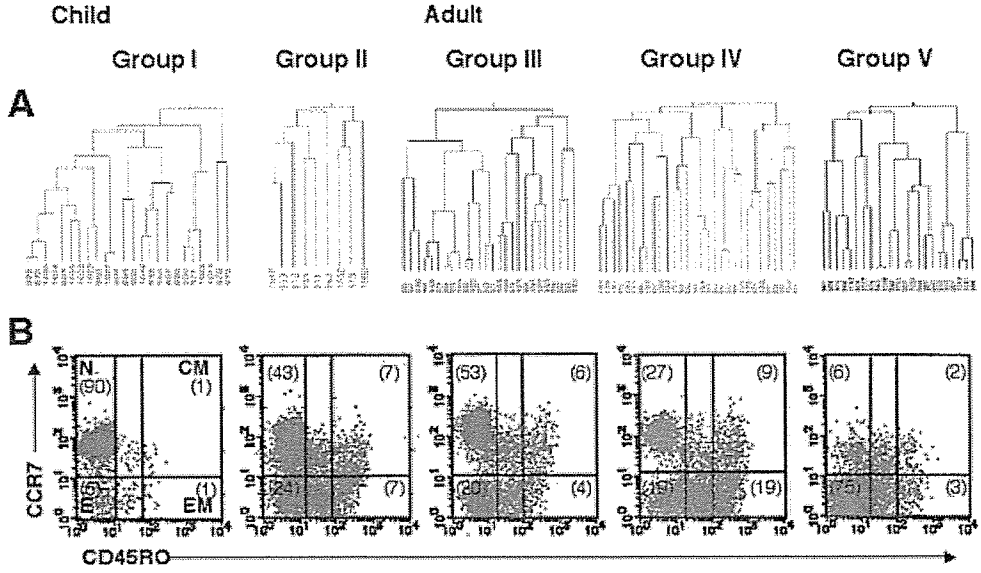

Figure 1. (A) Dendrogram of hierarchical clustering. (B) CD45RO and CCR7 are coexpressed on the subsets of peripheral blood $CD8^+$ T cells. Lymphocytes were stained with monoclonal antibodies to CD45RO and CCR7, which identified 4 subsets of $CD8^+$: 1 naive (N) ($CD45RO^-CCR7^+$); and 3 memory subsets,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

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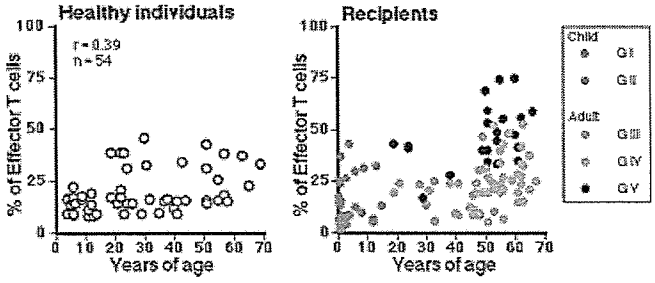

Figure 2. Changes in the proportion of circulating effector T cells with age in healthy individuals and transplant recipients. Each group is specified by a color marker: red, Group I; green, Group II; blue, Group III; brown, Group IV; and black, Group V.

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 β1 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