and/or spleen, or hind leg paralysis. GVHD death was defined as the absence of leukemia and by the presence of clinical signs of GVHD, assessed by using a clinical scoring system. Animals surviving beyond the observation period of BMT were sacrificed, and the spleen and liver were harvested for histological evaluation to determine leukemia-free survival.

Flow cytometric analysis. The mAbs used were FITC-, PE-, PerCP-, Cy5.5-, or APC-conjugated anti-mouse CD5.1, CD8, CD45.1, CD45.2, CD69, and PD-1 (BD Biosciences). Cells positive for 7-amino-actinomycin D (BD Biosciences) were excluded from the analysis. For the analysis of donor T cell apoptosis, the cells were stained with Annexin V (MBL). For intracellular IFN-γ staining, the splenocytes were incubated for 4 hours with leukocyte activation cocktail and BD GolgiPlug (BD Biosciences) at 37°C. Then, the cells underwent permeabilization with a BD Cytofix/Cytoperm solution (BD Biosciences) and were stained with FITC-conjugated anti–IFN-γ mAbs (BD Biosciences). For intracellular CTLA-4 staining, cells were stained with PE-conjugated anti–CTLA-4 mAbs (eBioscience). At least 5,000 live events were acquired for the analysis using a FACSCalibur flow cytometer (BD Biosciences).

CTL assay. Splenocytes were removed from chimeric recipients 14 days after BMT, and the mononuclear cells were then separated by density gradient centrifugation. The percentage of $CD8^+$ cells in this fraction was determined by flow cytometry, and counts were normalized for $CD8^+$ cell numbers. Tumor targets, 2×10^6 P815 or EL4, were labeled with 100 μCi of ^{51}Cr sodium salt (Amersham Biosciences) for 2 hours. After washing 3 times, the labeled targets were resuspended in 10% FCS in RPMI and plated at 10^4 cells per well in U-bottom plates (Corning-Costar Corp.). Allogeneic splenocyte preparations, as described above, were added to quadruplicate wells at varying effector-to-target ratios and incubated for 4 hours. Maximal and background release were determined by adding 1% SDS and media alone to the targets, respectively. ^{51}Cr activity in the supernatants collected 4 hours later was determined using a Wallac 1470 WIZARD Gamma Counter (Wallac Oy), and lysis was expressed as a percentage of maximum: percentage of specific lysis = 100 (sample count − background count/maximum count − background count).

Quantitative real-time PCR. Total RNA was isolated from the frozen liver using ISOGEN (Nippon Gene). cDNA was synthesized from 150 μg RNA using a QuantiTect Reverse Transcription Kit (QIAGEN). *Pdl1* mRNA levels were quantified by real-time PCR using the 7500 Real-Time PCR System (Applied Biosystems). TaqMan Universal PCR MasterMix, primers, and the fluorescent TaqMan probe specific for murine PD-L1 (Mm00452054-m1) and a house keeping gene, mGAPDH (Mm99999915-g1), were purchased from Applied Biosystems. The standard was obtained using RNA extracted from syngeneic controls.

Immunohistochemistry. For immunohistochemical analysis, isolated livers were frozen in Tissue-Tek (Sakura Finetek), and 5-μm cryostat sections were prepared. Slides were fixed in 100% acetone and air dried. Endogenous peroxidase activity was blocked with peroxidase blocking reagent (Dako). The sections were incubated with purified rat anti-mouse PD-L1 mAb (clone MIH5; eBiosciences). The primary Abs were detected using the Histofine Simple Stain Mouse MAX PO (Rat) kit and DAB solution (Nichirei). The images were captured using an Olympus BH2 microscope with a Nikon DS-5M color digital camera (Nikon), controlled by Nikon ATC-2U software version 1.5. An Olympus ×10/20 ocular lens and a ×20/0.46 NA objective lens were used. Images were cropped using Adobe Photoshop (Adobe Systems) and were composed using Adobe Illustrator.

Statistics. We used the Kaplan-Meier product-limit method to obtain survival probability and the log-rank test to compare survival curves. The Mann-Whitney *U* test was used to analyze the clinical scores. A *P* value less than 0.05 was considered statistically significant.

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Hepatic toxicity and prognosis in hepatitis C virus–infected patients with diffuse large B-cell lymphoma treated with rituximab-containing chemotherapy regimens: a Japanese multicenter analysis

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Hepatic toxicity and prognosis in hepatitis C virus–infected patients with diffuse large B-cell lymphoma treated with rituximab-containing chemotherapy regimens: a Japanese multicenter analysis

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The influence of hepatitis C virus (HCV) infection on prognosis and hepatic toxicity in patients with diffuse large B-cell lymphoma in the rituximab era is unclear. Thus, we analyzed 553 patients, 131 of whom were HCV-positive and 422 of whom were HCV-negative, with DLBCL treated with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (RCHOP)–like chemotherapy. Survival outcomes and hepatic toxicity were compared according to HCV infection. The median follow-up was 31 and 32 months for patients who were HCV-positive and HCV-negative, respectively. HCV infection was not a significant risk factor for prognosis (3-year progression-free survival, 69% vs 77%, $P = .22$; overall survival, 75% vs 84%, $P = .07$). Of 131 patients who were HCV-positive, 36 (27%) had severe hepatic toxicity (grade 3-4), compared with 13 of 422 (3%) patients who were HCV-negative. Multivariate analysis revealed that HCV infection was a significant risk factor for severe hepatic toxicity (hazard ratio: 14.72; 95% confidence interval, 6.37-34.03; $P < .001$). An exploratory analysis revealed that pretreatment transaminase was predictive of severe hepatic toxicity. HCV-RNA levels significantly increased during immunochemotherapy ($P = .006$). These results suggest that careful monitoring of hepatic function and viral load is indicated during immunochemotherapy for HCV-positive patients. (*Blood*. 2010; 116(24):5119-5125)

Introduction

Many epidemiologic studies have demonstrated an association between hepatitis C virus (HCV) infection and non-Hodgkin lymphoma, suggesting that HCV plays a role in the development of this malignancy.[1-8] Fewer data are available for patients who are HCV-positive with diffuse large B-cell lymphoma (DLBCL), as low-grade marginal zone lymphoma is the most common lymphoma subtype associated with HCV infection.[3,8] Thus, studies comparing DLBCL outcomes based on HCV infection are extremely rare, and the prognostic value of HCV infection remains controversial, because of heterogeneity in histology and treatment strategies.[9,10] Several series have shown good tolerance to standard chemotherapy for lymphoma patients who are infected with HCV.[9-11] However, these studies were conducted in the pre-rituximab era.

Although the cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) regimen has been the mainstay treatment for aggressive lymphomas for several decades,[12] treatment outcomes have improved significantly since the introduction of rituximab (an anti-CD20 chimeric antibody) in both young and elderly patients.[13-15] Since the introduction of rituximab, several prognostic factors have been reevaluated in DLBCL patients,[16-18] but the prognostic value of HCV infection in rituximab combination chemotherapy has not been well established. In addition, hepatitis B virus (HBV) reactivation is a well-documented complication that occurs frequently after introduction of rituximab.[19,20] However, no large-scale study has investigated the influence of HCV infection on hepatic toxicity in patients with DLBCL treated with rituximab-containing chemotherapy.

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We conducted a multicenter retrospective analysis to compare the prognosis and hepatic toxicity of untreated patients with DLBCL uniformly receiving rituximab plus CHOP-like chemotherapy according to HCV infection.

Methods

Patients

We collected medical information from patients who were HCV-positive with DLBCL, who received rituximab plus CHOP (RCHOP)-like therapy as a first-line treatment at 32 participating Japanese institutions between January 2004 and April 2008, and who were followed until March 2009. We also collected medical information from patients who were HCV-negative, treated within 2 months of the treatment start date for each patient who was HCV-positive. This study protocol was approved by the institutional review boards of each participating institute and complied with all provisions of the Declaration of Helsinki. DLBCL was diagnosed by an expert hematopathologist at each institute, based on the World Health Organization classification.[21] Patients were included if they were more than 20 years old, and an HCV infection test was performed before treatment. Both de novo DLBCL and transformed DLBCL (t-DLBCL) from low-grade B-cell lymphomas were included. Patients were excluded if they were positive for hepatitis B surface antigen (HBsAg) or for human immunodeficiency virus-1 or -2 antigens. Patients with primary central nervous system lymphoma, primary testicular lymphoma, and intravascular large-cell lymphoma (IVL) were also excluded.

In total, 590 patients, including 136 with and 454 without HCV infection, were registered. Then, 37 patients were excluded for the following reasons: HBsAg-positive (n = 2), non–rituximab-containing regimen (n = 2), absence of final outcome data (n = 1) in patients who were HCV-positive; except the definitive period corresponding to each patient who was HCV-positive (n = 26), regimens other than RCHOP-like (n = 3), HBsAg-positive (n = 2), and IVL (n = 1) in patients who were HCV-negative. In total, 553 patients were eligible; 131 patients (23.7%) were HCV-positive, whereas 422 (76.3%) were HCV-negative.

Clinical and laboratory information, including antibodies to HBsAg (anti-HBs) and hepatitis B core antigen (anti-HBc), was available at the time of diagnosis. HCV infection was defined as the detection of anti-HCV antibodies with commercially available second- or third-generation immunoassay kits (Monolisa anti-HCV Plus, Sanofi Diagnostics Pasteur; and Axsym HCV Version 3.0, Abbott Laboratories).

Treatment and response assessment

All DLBCL patients with HCV infection during the definitive period received immunochemotherapy. Chemotherapy regimens included RCHOP, rituximab plus cyclophosphamide, therarubicin, vincristine, and prednisone, and rituximab plus cyclophosphamide, epirubicin, vincristine, and prednisone. Disease stage was evaluated using the Ann Arbor staging system. Liver and spleen involvement was diagnosed by imaging lymphoma invasion, such as nodular lesions or heterogeneous concentrations. Chemotherapy sensitivity was defined according to standard volume criteria, using computed tomography (CT) and positron emission tomography (PET)/CT with [^{18}F]-fluorodeoxyglucose imaging.[22,23]

Liver function tests and HCV viral markers

In all patients enrolled, pretreatment levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and their highest levels up to 6 months after completing immunochemotherapy were collected for analysis. Hepatic toxicity was defined by the National Cancer Institute of Canada criteria, and severe hepatic toxicity was defined as an increase in transaminase levels (AST or ALT, grade 3 or 4; $> 5.0 \times$ ULN). To assess impaired hepatic synthesis, serum total bilirubin (T-Bil), albumin (Alb), prothrombin time, and platelet counts were collected at the time of DLBCL diagnosis and at 1 and 6 months after treatment. Serum HCV RNA load was determined by quantitative reverse-transcription polymerase chain reaction (detection value of 5-5000 KIU/mL; Amplicor HCV Monitor Test, Version 2.0, Cobas-High-Range assay, Roche Diagnostics).

Statistical analyses

Progression-free survival (PFS) was calculated from the treatment initiation date to the date of documented disease progression, relapse, or the end date of the study. Non–lymphoma-related deaths were censored for PFS. Overall survival (OS) was calculated from the treatment initiation date until death from any cause or the last follow-up. If the stop date was not reached, the data were censored at the date of the last follow-up evaluation. Survival curves were created by the Kaplan-Meier method, and overall differences were compared using the log-rank test. Multivariate analysis was performed using a Cox proportional hazard model to estimate the independent impact of HCV infection on survival and severe hepatic toxicity. In patients who were HCV-positive, risk factors for severe hepatic toxicity were also evaluated by multivariate analysis. Changes in transaminase levels with respect to HCV infection were compared using the Kruskal-Wallis test. Intraindividual changes in serum HCV-RNA levels were assessed using the Wilcoxon signed-rank test. We evaluated impaired hepatic synthesis according to HCV infection using a univariate linear regression model. The basic characteristics and outcomes of each group (HCV-positive and -negative) were compared using the χ^2 test, t test, and the Mann-Whitney U test, as appropriate. All statistical tests were 2-sided, and the differences were deemed to be statistically significant if $P < .05$. Data were analyzed using the Stata software Version 10 (StataCorp LP).

Results

Patient characteristics

Patient characteristics are listed in Table 1. The median age of all patients was 68 years (range, 20-92 years). Before treatment, patients who were HCV-positive were older ($P < .001$), had more frequently elevated lactate dehydrogenase levels ($P = .002$), more than 2 extranodal sites ($P = .02$), spleen involvement ($P = .001$), and a higher international prognostic index (IPI) ($P = .01$) than patients who were HCV-negative. There was no difference in the occurrence of t-DLBCL according to HCV infection.

Of the patients who were HCV-positive at the time of DLBCL diagnosis, 57 (43%) and 20 (15%) patients were known to have chronic hepatitis (CH) and liver cirrhosis (LC), respectively. Hepatocellular carcinoma (HCC) complications were detected in 7 patients (5%) before immunochemotherapy. The diagnosis of HCC was made by CT or magnetic resonance imaging in 3 patients and by biopsy in 4 patients. Seven patients with CH and one patient with LC were HB serology-positive. One HB serology-positive patient was given prophylactic lamivudine treatment during immunochemotherapy. Twelve patients (9%) had a history of anti-HCV therapy before DLBCL treatment, 2 patients continued anti-HCV therapy during immunochemotherapy, and 5 patients restarted anti-HCV therapy after immunochemotherapy.

Survival analysis

The median follow-up time was 31 months (range, 4-42 months) for patients who were HCV-positive and 32 months (range, 5-51 months) for those who were HCV-negative. Complete remission (CR) or uncertain CR rates were 81% and 83% in HCV-positive and HCV-negative patients, respectively. No significant difference was observed in PFS according to HCV infection (3-year PFS, 69% vs 77%, $P = .22$). The OS tended to be worse in patients who were HCV-positive than in those who were HCV-negative (3-year OS, 75% vs 84%, $P = .07$; Figure 1A-B). The PFS and OS rates at 3 years were 56% and 64% for high IPI or

Table 1. Comparison of HCV-positive with HCV-negative patients

	HCV-positive (n = 131), n (%)	HCV-negative (n = 422), n (%)	*P*
Median age, y (range)	70.4 (42-86)	64.3 (20-92)	< .001
Sex, male/female	79/52	228/194	.21
LDH > normal	81 (62)	196 (46)	.002
PS > 1	16 (12)	40 (9)	.47
Stage			.48
I	28 (21)	92 (22)	
II	39 (30)	130 (31)	
III	20 (15)	84 (20)	
IV	44 (34)	116 (27)	
Extranodal sites > 1	36 (27)	75 (18)	.02
IPI: H/I, H	53 (40)	139 (33)	.01
BM involvement	12 (9)	38 (9)	.96
Spleen involvement	24 (18)	35 (8)	.001
Liver involvement	12 (9)	25 (6)	.20
t-DLBCL	5 (4)	11 (3)	.82
FL	3	5	
MZBCL	2	6	
HBsAb-positive	7/59 (12)	13/135 (10)	.24
HBcAb-positive	11/22 (50)	9/57 (16)	.03
Treatment			.12
RCHOP	96 (73)	339 (80)	
RTHPCOP	31 (24)	71 (17)	
RCEOP	4 (3)	12 (3)	
Baseline transaminase			.48
Grade 0-1	122 (93)	415 (98)	
Grade 2	7 (5)	3 (1)	
Grade 3	2 (2)	4 (1)	
Outcome of patients			
Died of lymphoma	14 (11)	45 (11)	.87
Died of hepatic failure	6 (5)	1 (0.2)	< .001
Died of other causes	4 (3)	7 (2)	.76
Hepatic toxicity			
Grade 3-4	36 (27)	13 (3)	< .001

LDH indicates lactate dehydrogenase; PS, ECOG performance status; H/I, high-intermediate; H, high; BM, bone marrow; t-DLBCL, transformed diffuse large B-cell lymphoma; FL, follicular lymphoma; MZBCL, marginal zone B-cell lymphoma; HBsAb, antibody to hepatitis B surface antigen; HBcAb, antibody to hepatitis B core antigen; RCHOP, rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone; RTHPCOP, rituximab plus cyclophosphamide, teralbicin, vincristine, and prednisone; and RCEOP, rituximab plus cyclophosphamide, epirubicin, vincristine, and prednisone. Grade was defined by the National Cancer Institute of Canada criteria.

high-intermediate and 81% and 88% for low IPI or low-intermediate, respectively. In these cases there were significant differences in the PFS and OS rates (both $P < .001$). Cox multivariate analysis showed that older age and advanced stage had significant adverse effects on OS. In contrast, HCV infection was not associated with poor PFS or OS (Table 2). According to the cause-specific analysis, survival rates for lymphoma did not significantly correlate with HCV infection (3-year, 84% vs 86%; $P = .80$).

Hepatic toxicity

The pretreatment transaminase levels were not significantly different in patients with HCV infection (Table 1). Of the 131 patients who were HCV-positive, 36 (27%) had severe hepatic toxicity, compared with 3% of those who were HCV-negative. Multivariate analysis revealed that HCV infection was a significant risk factor for severe hepatic toxicity (hazard ratio [HR]: 14.72; 95% confidence interval [CI], 6.37-34.03; $P < .001$; supplemental Table 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). The changes in AST and ALT levels with respect to HCV infection were significant (both $P < .001$; Figure 2A-B). The median time from treatment initiation to the development of severe hepatic toxicity was 103 days

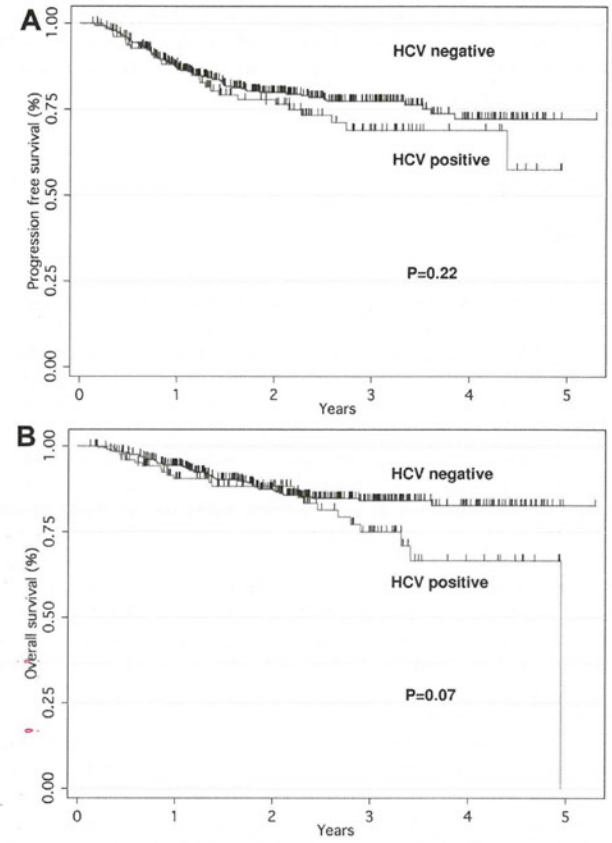

Figure 1. PFS and OS curves for patients with DLBCL treated with RCHOP according to HCV infection. PFS (A) and OS (B) curves based on patients who were HCV-positive (n = 131) versus HCV-negative (n = 422).

Table 2. Multivariate analysis of survival

	PFS			OS		
Characteristic	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
HCV infection						
Negative	1.00	Ref		1.00	Ref	
Positive	0.97	0.59-1.60	NS	1.22	0.69-2.15	NS
Sex						
Male	1.00	Ref		1.00	Ref	
Female	1.47	0.98-2.22	NS	1.44	0.88-2.37	NS
Age, y						
20-60	1.00	Ref		1.00	Ref	
> 60	1.02	1.01-1.04	.01	1.03	1.01-1.06	.007
LDH						
Normal	1.00	Ref		1.00	Ref	
> Normal	1.16	0.72-1.87	NS	1.37	0.77-2.43	NS
PS						
0-1	1.00	Ref		1.00	Ref	
2-4	1.28	1.04-1.59	.02	1.16	0.89-1.51	NS
Extranodal sites						
0 or 1	1.00	Ref		1.00	Ref	
2	0.77	0.45-1.30	NS	0.87	0.45-1.68	NS
Stage						
I, II	1.00	Ref		1.00	Ref	
III, IV	1.64	1.28-2.11	< .001	1.67	1.20-2.32	.002

Ref indicates reference; and NS, not significant.

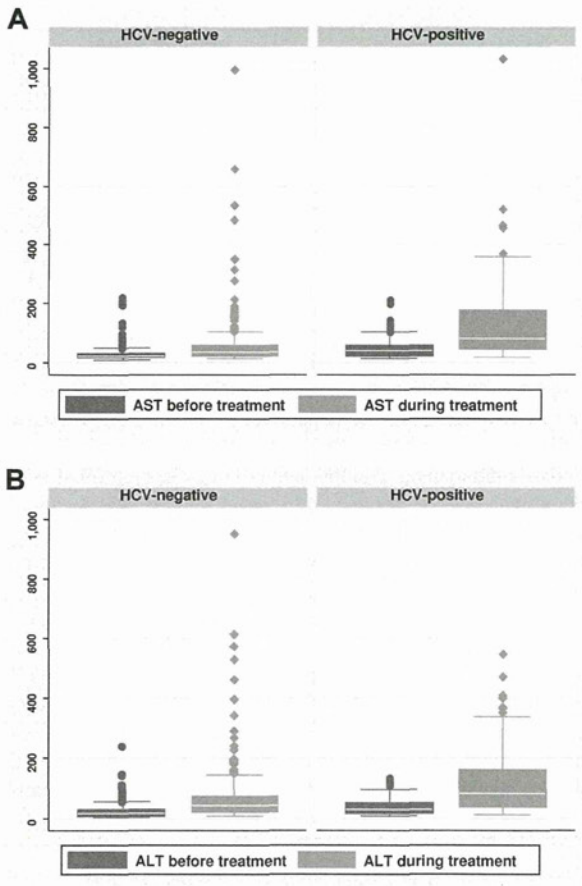

Figure 2. The changes of AST and ALT levels between before treatment levels and highest levels up to 6 months after completing immunochemotherapy. The changes in AST (A) and ALT (B) levels with respect to HCV infection were significant (both $P < .001$).

(range, 8-320 days). In those who were HCV-positive, pretreatment transaminase levels were significant risk factors (HR, 6.42; 95% CI, 1.11-37.12; $P < .001$) for severe hepatic toxicity among baseline characteristics (supplemental Table 2). In HCV-positive patients, severe hepatic toxicity was observed in 6 (33%) of 18 patients and 30 (27%) of 113 patients with and without omission of prednisone, respectively ($P = .82$).

Severe hepatic toxicity was not associated with poor PFS or OS in patients who were HCV-positive ($P = .56$ and $P = .51$, respectively). However modification of the scheduled dose was required for 16 patients because of severe hepatic toxicity, and chemotherapy was withdrawn for 6 patients, including 2 who died because of disease progression (Table 3). The remaining 4 patients stopped immunochemotherapy; however, they received radiation therapy after their liver function normalized or they discontinued treatment, and they were alive and in remission.

Changes in serum T-Bil, Alb, platelet counts, and prothrombin time showed no difference with regard to HCV infection status (supplemental Figure 1). Progression of hepatic complications during treatment occurred in 4 cases; progression from CH to LC, and from LC to HCC, occurred in 2 patients each.

Of the 24 deaths in patients who were HCV-positive, 14 were caused by disease progression, and 6 were caused by hepatic failure (Table 4). The 4 remaining deaths were caused by 2 cardiovascular and 2 brain vascular events. Among HCV-positive patients, HCC was predictive of death from hepatic failure (supplemental Table 3).

In total, 36 HCV-positive patients developed severe hepatic toxicity. At the time they developed severe hepatic toxicity, 29 patients were negative for either HBsAg or HBV-DNA, and 7 patients had no data about HBsAg or HBV-DNA. None of the 36 patients received any anti-HBV viral therapy, such as lamivudine.

HCV viral load

HCV-RNA levels were collected during pretreatment (n = 79), at the highest levels during the period of treatment initiation to 1 mo after treatment completion (n = 47), and 1-6 months after treatment (n = 44). HCV-RNA levels for all 3 time points were available from 34 patients. Mean HCV-RNA levels at the 3 time points as determined from those samples were 1001 (95% CI, 522-1481), 3187 (2148-4232), and 1986 (1145-2827) KIU/mL, indicating that HCV-RNA increased significantly during immunochemotherapy ($P = .006$) but then decreased afterward ($P = .003$; Figure 3). In 2 patients who received antiviral therapy during immunochemotherapy, HCV-RNA levels increased after the initiation of immunochemotherapy. In 3 cases where HCV-RNA was under the threshold level (< 5 KIU/mL) before treatment, HCV-RNA increased, more than 100-fold, and 2 patients developed severe hepatic toxicity (Table 5).

Discussion

This study showed that prognosis did not differ according to HCV infection. In sharp contrast, the incidence of severe hepatic toxicity was high in HCV-positive patients, and HCV infection was determined to be a strong risk factor for this adverse effect. These findings were not consistent with previous reports demonstrating good tolerance for HCV infection in the pre-rituximab era.[8,10,11,24] This is the first large study to show the influence of HCV infection on prognosis and hepatic toxicity in patients with DLBCL in the rituximab era.

A previous report showed that HCV-positive patients with DLBCL exhibited worse OS, but not event-free survival; however, this study included a very small number of HCV-positive patients

Table 3. Details and outcomes of 6 patients withdrawn from immunochemotherapy due to severe hepatic toxicity

Case	Age, y	Sex	Stage	Hepatic disease at baseline	AST/ALT at baseline, IU/L	AST/ALT peak level, IU/L	No. of cycles of treatment before withdrawal	Outcome
1	76	F	III	LC	101/73	236/114	4	Died of lymphoma progression
2	78	F	III	CH	38/25	1033/340	1	Died of lymphoma progression
3	71	F	I	LC	90/82	289/128	1	Alive
4	59	M	IV	CH	31/17	211/284	4	Alive
5	74	F	IV	Normal	50/29	274/302	3	Alive
6	76	M	II	Normal	19/25	522/550	3	Alive

Table 4. Details of 6 patients who died due to hepatic failure

Case	Age, y	Sex	Stage	Treatment	Hepatic disease at baseline	AST/ALT, IU/L		HCV-RNA, KIU/mL	
						At baseline	Peak level	At baseline	Peak level during treatment
1	75	M	I	RCEOP	LC	91/63	136/102	ND	1000
2	74	F	IV	RTHPCOP	HCC	29/16	58/49	4400	5000
3	68	F	II	RTHPCOP	CH	118/76	203/113	510	1600
4	71	F	III	RCHOP	HCC	34/41	242/139	470	ND
5	64	M	IV	RCHOP	HCC	27/10	97/32	ND	ND
6	76	M	III	RTHPCOP	HCC	46/40	111/107	434	21 300

ND indicates not done.

(n = 23) and was based on short-term follow-up.[9] Another large scale study of DLBCL demonstrated that the 5-year PFS was 51% and OS was 72%, but the relationship between HCV infection and outcome in this study remained unclear, because of the lack of an HCV-negative control group.[10] In addition, these studies were performed in the pre-rituximab era, and no large-scale study has compared the outcome for DLBCL treated with rituximab according to HCV infection. The present study found that HCV-positive patients exhibited more aggressive baseline behavior, consistent with previous reports. These patients also exhibited borderline poor OS by univariate analysis. However, it is important to note that no significant difference was observed in CR rates or PFS according to HCV infection, and multivariate analysis revealed that HCV infection was not a significant risk factor for prognosis.

In the pre-rituximab era, several series showed good tolerance to standard chemotherapy for HCV-infected patients with lymphoma or other hematologic malignancies, with greater than grade 3 liver dysfunction in 10%-18% of patients, and grades 1-4 liver dysfunction in 24% of patients.[10,11,25] In contrast, Besson et al found a higher incidence of toxic deaths among HCV-positive DLBCL patients compared with HCV-negative patients, although this study was based on patients treated with more aggressive chemotherapy than the standard CHOP regimen.[9] A recent analysis also showed an increase in hepatic toxicity in HCV-positive patients with B-cell lymphoma.[26] However, this study included no control group of HCV-negative patients and included heterogeneous treatment strategies and several lymphoma subtypes. In addition, previous series did not exclude HBsAg-positive patients, known to be a high-risk population for severe hepatic injury or fulminant hepatitis.[9-11,25,26] Our study showed that the incidence in severe hepatic toxicity in HCV-positive patients was significantly higher than that of HCV-negative patients (27% vs 3%; $P < .001$), and that these hepatic toxicities led to modification and discontinuation of immunochemotherapy, resulting in lymphoma progression. Careful monitoring of hepatic function should, thus, be recommended for HCV-positive patients, particularly those with high levels of pretreatment transaminase.

Recent case reports have suggested that combined use of rituximab and chemotherapy poses an additional risk for exacerbation of HCV infection.[27-29] However, HCV replication during chemotherapy has not been well characterized. In the present study, monitoring of HCV viral load demonstrated a marked enhancement in HCV replication, and it is suggested that increased HCV expansion results in severe hepatic toxicity. Thus, HCV viral load should also be carefully monitored in HCV-positive patients who receive immunochemotherapy, although this finding must be considered with caution because of the small sample size. In addition, this study revealed that HCV-RNA levels could increase during immunochemotherapy, even though HCV-RNA levels were extremely low (< 5 KIU/mL) before treatment.

Recent reports have indicated that HBsAg-negative/anti-HBc or HBs-positive patients could also develop HBV reactivation after rituximab treatment.[30,31] Among the HCV-positive patients analyzed in the present study for HBV-DNA and HBsAg at the time of hepatic dysfunction, none was positive, and in all patients, severe hepatic toxicity improved with no anti-HBV treatment. In addition, neither anti-HBs– nor anti-HBc–positive patients were found to be in a significant risk group for severe hepatitis (anti-HBs; HR, 0.42; 95% CI, 0.03-5.81, anti-HBc; HR, 0.23; 95% CI, 0.01-1.98). Although we could not completely rule out the possibility of HBV reactivation, we suggest that HCV rather than HBV contributed to severe hepatic toxicity in the present cohort infected with HCV.

In accordance with previous reports,[9-11] HCV-positive patients in the present unmatched study had more aggressive tumor behavior at baseline and more frequent spleen involvement. The mechanisms underlying the association of HCV infection with aggressive tumor behavior are not well understood. Further studies of the biological features of HCV-infected DLBCL, such as germinal center phenotype, are necessary.

HCV infection results in a long-term risk for progression to LC and HCC. One study reported a rapid progression of hepatitis C in patients with humoral immunodeficiency disorders,[32] and an increased rate for liver fibrosis progression was observed in HCV-infected patients who received an allogeneic bone marrow transplant.[33] A possible explanation for the genesis of cirrhosis could be an immune imbalance or impaired regulation of B and T cells.[32,33] We showed that hepatic synthesis after treatment was not affected by HCV infection. However, hepatic disease progressed in 4 patients, and HCC was found to significantly increase the risk of death of hepatic failure, even during short-term observation. Further studies are necessary to clarify the contribution of rituximab to the risk of progressive liver damage.

Table 5. Details and outcomes of the 3 DLBCL patients with HCV-RNA below threshold level before immunochemotherapy

Case	Age, y	Sex	HCV-RNA level, KIU/mL			AST/ALT, IU/L			Outcome/follow-up time, (mo)
			At baseline	Peak level during treatment	At last evaluation	At baseline	Peak level during treatment	At last evaluation	
1	59	M	< 5	3960	79	19/12	212/71	44/18	Alive/6
2	77	F	< 5	1200	< 5	40/15	156/125	33/34	Alive/28
3	74	F	< 5	590	270	50/29	274/302	34/29	Alive/28

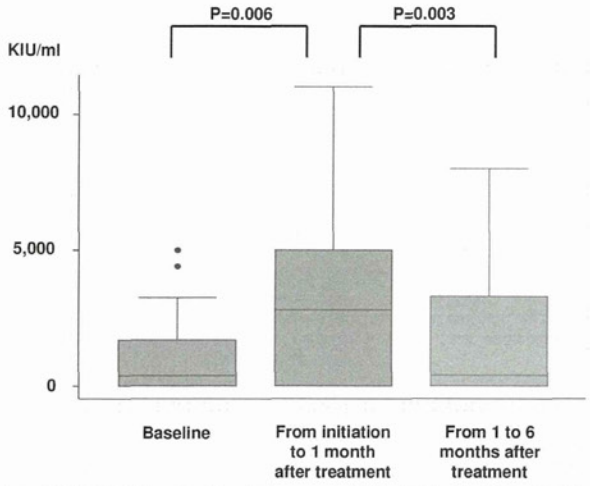

Figure 3. HCV-RNA levels at 3 points were available in 34 patients; pretreatment level, highest levels from initiation of treatment to 1 month after completing treatment, and from 1 to 6 months after treatment. HCV-RNA significantly increased during immunochemotherapy ($P = .006$) and then decreased after treatment ($P = .003$).

Although we believe that our data provide novel information about rituximab-treated patients who are HCV-positive with DLBCL, some limitations of these findings should be discussed. First, because this retrospective study included enrollment from many institutions, unrecognized biases might have been introduced. Second, we did not register all patients who were HCV-negative during the study period, but only those who were treated over the same time period as patients who were HCV-positive at each institution. This might have caused case-selection bias. Finally, we did not define the timing of immunochemotherapy withdrawal because of hepatic toxicity as we believe that the decision to terminate immunochemotherapy must be made by the treating physician.

In conclusion, our study highlighted a high incidence of severe hepatic toxicity in patients who were HCV-positive, so hepatic function should be carefully monitored in patients who are HCV-positive and receive immunochemotherapy. Well-designed studies will be necessary to determine whether early detection and prevention of HCV replication would provide improved disease management for HCV infected patients receiving immunochemotherapy.

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Authorship

Contribution: D.E. and Y.M. designed research, performed research, analyzed data, and wrote the paper; N.N, M.K, K.I, J.T, S.K, M.O, M.Y., and A.T. designed and performed research; Y.T, K.S, A.M, K.M, A.S, M.M., and K.S. performed research; K.M. designed research, analyzed data, and wrote the paper; and T.K and T.M. designed research and gave final approval.

Conflict-of-interest disclosure: S.K. received research funding from Chugai Pharmaceutical Co Ltd; M.Y. is a medical advisor to Chugai Pharmaceutical Co Ltd; T.K. received honoraria from Chugai Pharmaceutical Co Ltd and Zenyaku Kogyo Co Ltd, and research funding from Zenyaku Kogyo Co Ltd. The remaining authors declare no competing financial interests.

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ORIGINAL REPORT

Phase II Study of SMILE Chemotherapy for Newly Diagnosed Stage IV, Relapsed, or Refractory Extranodal Natural Killer (NK)/T-Cell Lymphoma, Nasal Type: The NK-Cell Tumor Study Group Study

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ABSTRACT

Purpose
To explore a more effective treatment for newly diagnosed stage IV, relapsed, or refractory extranodal natural killer/T-cell lymphoma, nasal type (ENKL), we conducted a phase II study of the steroid (dexamethasone), methotrexate, ifosfamide, L-asparaginase, and etoposide (SMILE) regimen.

Patients and Methods
Patients with newly diagnosed stage IV, relapsed, or refractory disease and a performance status of 0 to 2 were eligible. Two cycles of SMILE chemotherapy were administered as the protocol treatment. The primary end point was the overall response rate (ORR) after the protocol treatment.

Results
A total of 38 eligible patients were enrolled. The median age was 47 years (range, 16 to 67 years), and the male:female ratio was 21:17. The disease status was newly diagnosed stage IV in 20 patients, first relapse in 14 patients, and primary refractory in four patients. The eligibility was revised to include lymphocyte counts of 500/μL or more because the first two patients died from infections. No treatment-related deaths were observed after the revision. The ORR and complete response rate after two cycles of SMILE chemotherapy were 79% (90% CI, 65% to 89%) and 45%, respectively. In the 28 patients who completed the protocol treatment, 19 underwent hematopoietic stem-cell transplantation. The 1-year overall survival rate was 55% (95% CI, 38% to 69%). Grade 4 neutropenia was observed in 92% of the patients. The most common grade 3 or 4 nonhematologic complication was infection (61%).

Conclusion
SMILE chemotherapy is an effective treatment for newly diagnosed stage IV, relapsed or refractory ENKL. Myelosuppression and infection during the treatment should be carefully managed.

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INTRODUCTION

Extranodal natural killer (NK)/T-cell lymphoma, nasal type (ENKL), is a lymphoma associated with the Epstein-Barr virus (EBV), which is much more common in Asia and Latin America than in Western countries.[1,2] More than two thirds of patients with ENKL have stage I or II disease in the upper aerodigestive tract.[3-6] The prognosis for localized ENKL has been improving as a result of the use of either concurrent chemoradiotherapy[7,8] or chemotherapy with sandwiched radiotherapy.[9] In contrast, most patients with newly diagnosed stage IV, relapsed, or refractory ENKL treated with conventional chemotherapy designed for aggressive lymphomas, such as cyclophosphamide, doxorubicin, vincristine, and prednisone, survive for less than a year.[6] The poor outcome is partly because ENKL tumor cells express P-glycoprotein, which results in tumor multidrug resistance.[10-12] There are a number of long-term survivors among patients with advanced-stage, relapsed, or refractory ENKL who have undergone hematopoietic stem-cell transplantation (HSCT).[13-15] However, patients who received HSCT in complete response (CR) showed better prognosis than those who received HSCT during non CR. Therefore, the development of an effective chemotherapy for these patients is an important initial step in improving treatment outcomes.

Table 1. SMILE Chemotherapy

Agent	Dose/d	Route	Day
Methotrexate	2 g/m^2*	IV (6 hours)	1
Leucovorin	15 mg × 4	IV or PO	2, 3, 4
Ifosfamide	1,500 mg/m^2	IV	2, 3, 4
Mesna	300 mg/m^2 × 3	IV	2, 3, 4
Dexamethasone	40 mg/d	IV or PO	2, 3, 4
Etoposide	100 mg/m^2*	IV	2, 3, 4
L-asparaginase (*Escherichia coli*)	6,000 U/m^2	IV	8, 10, 12, 14, 16, 18, 20
G-CSF		SC or IV	Day 6 to WBC > 5,000/μL

NOTE. Cycles were repeated every 28 days. Two courses were planned as the protocol treatment.
Abbreviations: G-CSF, granulocyte-colony stimulating factor; IV, intravenously; PO, orally; SC, subcutaneous injection; SMILE, steroid (dexamethasone), methotrexate, ifosfamide, L-asparaginase, and etoposide.
*The recommended dose was determined in the preceding phase I study.

To explore the possibility of more effective induction chemotherapy for NK-cell neoplasms, the NK-Cell Tumor Study Group, comprising Japanese and Asian hematologists, has formulated a novel chemotherapeutic regimen: steroid (dexamethasone), methotrexate, ifosfamide, L-asparaginase, and etoposide (SMILE). These agents are multidrug resistance independent and may be key drugs for NK-cell neoplasms or for EBV-associated disease. From the phase I trial of SMILE, the recommended doses of methotrexate and etoposide were determined.[16] The CR rate in the phase I trial was 50% (three of six eligible patients), and the overall response rate (ORR) was 67% (four of six patients). To further evaluate the efficacy of SMILE chemotherapy, we conducted a subsequent phase II study.

PATIENTS AND METHODS

Eligibility Criteria

Patients with newly diagnosed stage IV, relapsed, or refractory disease who had undergone first-line chemotherapy were eligible. Those with aggressive NK-cell leukemia were excluded because no patients with aggressive NK-cell leukemia had been enrolled in the prior phase I study.[16] Patients who had received autologous HSCT more than 12 months before registration were also eligible. The other inclusion and exclusion criteria for the study were the same as those for the prior phase I study.[16] Briefly, patients from 15 to 69 years of age with a performance status of 0 to 2, based on the Eastern Cooperative Oncology Group scale, and preserved organ functions were included. Neither chemotherapy nor radiotherapy was administered within 21 days before registration. Patients who had clinical symptoms of CNS involvement were excluded.

The pretreatment staging procedures included a physical examination, a bone marrow aspiration and/or biopsy, a chest radiograph, and a computed tomography scan of the nasal cavity, neck, chest, abdomen, and pelvis. An endoscopy of the upper gastrointestinal tract and a positron emission tomography scan were recommended but not mandatory.

After patient enrollment, hematoxylin-eosin–stained sections were histologically reviewed by the Central Pathology Review Board based on the WHO classification.[1] Immunohistochemical staining was performed at the central pathology office using formalin-fixed, paraffin-embedded sections with antibodies against CD3, CD20, CD56, perforin, and granzyme B. In addition, in situ hybridization for EBV-encoded small RNA-1 was performed.

Registration of patients was conducted by facsimile between the participating physicians and the Center for Supporting Hematology-Oncology Trials Data Center (Nagoya, Japan). The study was approved by both the protocol review committee and the institutional review board of each institution. Written informed consent was obtained from all of the patients. The study was registered to the University Hospital Medical Information Network Clinical Trials Registry.

Treatment

SMILE chemotherapy was administrated as indicated in Table 1. On the basis of the results of the phase I trial,[16] administration of granulocyte colony-stimulating factor was mandatory from day 6 and discontinued if the leukocyte count exceeded 5,000/μL after the nadir phase. Antibiotic prophylaxis of sulfamethoxazole-trimethoprim was recommended. The criteria for the initiation of a second course of SMILE were as follows (1): a total of 4 weeks or more had passed since the prior course; (2) all of the following were achieved at least 1 day before the second course of SMILE: a leukocyte count of $\geq$ 2,000/μL, a platelet count of $\geq$ 100,000/μL, AST and ALT levels $\leq$ 5$\times$ the upper limit of normal, total bilirubin of $\leq$ 2.0 mg/dL, or serum creatinine of $\leq$ 1.5 mg/dL; and (3) there were no other symptoms or complications that were not suitable for the initiation of a second course. If there was no recovery 4 weeks after the day of the scheduled second course, the protocol treatment was terminated. Two courses of SMILE chemotherapy were planned for the protocol treatment. After the planned two courses, patients could undergo additional courses of SMILE and/or other chemotherapy, with or without autologous/allogeneic HSCT. The decision was made according to the discretion of treating physicians mainly on the basis of the patient's age, conditions, and the availability of HSC donors.

Response and Toxicity Criteria

The responses were assessed by the Central Imaging Review Board according to criteria modified from the WHO response criteria[17] that were also used in the prior phase I study of SMILE chemotherapy.[16] All of the examinations for restaging were done within 4 to 6 weeks (from day 22 to day 42) of the second course of SMILE. Because ENKL frequently occurs in the nasal/paranasal sites and leaves scar or necrotic tissue, it is sometimes difficult to determine whether or not a patient strictly attains CR using the WHO response criteria[17] or the International Workshop criteria.[18] Therefore, in this trial, CR was defined as the complete disappearance of all objective signs of disease, including enlarged lymph nodes or hepatomegaly and splenomegaly at the restaging. Partial response (PR) was defined as at least a 50% reduction of tumor volume without the occurrence of new lesions at the restaging. Progressive disease was defined as a greater than 25% increase in the sum of tumor lesions or the emergence of one or more new lesion(s) or clinical symptoms that indicate disease progression, such as "B" symptoms or elevated serum lactate dehydrogenase levels. No response was defined as any response that did not fall into the other defined categories. If a patient died before day 42 of the second course of SMILE and could not undergo the defined restaging procedure, the patient's response was recorded as early death. The ORR rate was defined as the proportion of all patients who were able to be evaluated for response who experienced CR or PR.

Toxicity was graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. In cases of grade 4 thrombocytopenia,

doses of methotrexate, ifosfamide, and etoposide were reduced to two thirds of their previous levels in the second course. L-asparaginase was discontinued if it induced grades 3 or 4 allergic reactions/hypersensitivity, pancreatitis, or hypotension. If L-asparaginase induced grades 1 or 2 allergic reactions/hypersensitivity, the dose of L-asparaginase was reduced by half. In this case, the use of prednisone at a dose of 1 mg/kg/d was permitted. L-asparaginase was stopped if grade 4 thrombocytopenia or grade 3 nonhematologic toxicity was observed. In the cases for which the first course of L-asparaginase was discontinued, L-asparaginase was readministered if the patient recovered from grade 4 thrombocytopenia or grade 3 nonhematologic toxicity. If the concentration of methotrexate exceeded 1×10^{-7} mol/L 72 hours after the administration during the first course, the dose of methotrexate in the second course was reduced to two thirds.

Statistical Analysis

The primary end point was an ORR after two courses of SMILE chemotherapy. The secondary end points were CR rate after two courses of SMILE chemotherapy, 1-year overall survival (OS), response of the subgroup, or toxicity. The expected ORR was estimated to be 60%, and the threshold ORR was estimated to be 35%, on the basis of our previous observations.[6,19] With a statistical power of 80% and a one-sided, type I error of 5%, the number of eligible patients required for this study was calculated to be 25 using a binomial analysis method. The projected sample size was 28 patients, with an accrual of 3 years and the expectation that 10% of patients would be deemed ineligible.

OS was defined as the time from registration until death from any cause or until the date of the last follow-up for the patients who survived. Survival estimates were calculated using the Kaplan-Meier method, and the hazard ratio (HR) was estimated using a Cox regression. All analyses were performed using STATA SE 10 software (STATA, College Station, TX).

RESULTS

Patient Characteristics

As a result of an excellent accrual, the study protocol was revised to increase the statistical power from 80% to 90% in March 2009. The projected number of patients for this study was increased from 28 to 38. Ultimately, 39 patients were enrolled from 19 institutions between July 2007 and October 2009. Histologic diagnosis of all patients except one was confirmed as ENKL by the Central Pathology Review Board. The single patient who was excluded from further analyses was judged to have CD56-positive rhabdomyosarcoma by the Central Pathology Review Board.

The baseline characteristics of 38 eligible patients are listed in Table 2. The median age was 47 years (range, 16 to 67 years), and the male:female ratio was 21:17. Twenty patients (53%) had newly diagnosed stage IV disease, 14 were in first relapse, and four were in primary refractory state. Two patients were treated with radiation alone as the initial therapy. Among the 16 patients who received chemotherapy as their first-line therapy, five patients were treated with anthracycline-containing chemotherapies, and 13 patients were treated with platinum-based regimens. Two patients were treated with chemotherapy containing both anthracycline and platinum.

Table 2. Baseline Patient Characteristics (N = 38)

Characteristic	No. of Patients	%
Age, years		
Median	47	
Range	16 to 67	
Sex		
Male	21	55
Female	17	45
Site(s) of involvement at diagnosis		
Upper aerodigestive tract	35	92
Extra-upper aerodigestive tract only	3	8
Disease state		
Newly diagnosed stage IV	20	53
First relapse	14	37
Refractory to the first-line treatment	4	11
Stage at enrollment		
IE or IIE	11	29
IIIE or IV	27	71
"B" symptoms present	18	47
Elevated serum LDH	16	42
Performance status		
0	21	55
1	12	32
2	5	13
Prior treatment		
None	20	53
Radiotherapy alone	2	5
Chemotherapy alone	3	8
Concurrent chemoradiotherapy	9	24
RT-DeVIC	6	
CCRT-VIPD or VIDL	2	
RT-CHOP	1	
Other combined modality therapies	4	11

Abbreviations: CCRT, concurrent chemoradiotherapy; DeVIC, dexamethasone, etoposide, ifosfamide, and carboplatin; LDH, lactate dehydrogenase; VIDL, etoposide, ifosfamide, dexamethasone, and L-asparaginase; VIPD, etoposide, ifosfamide, cisplatin, and dexamethasone.

Treatment

Twenty-eight patients (74%) completed the planned treatment. In two patients, the treatment was discontinued on day 4 because of methotrexate-associated encephalopathy and intestinal perforation owing to rapid tumor lysis. L-asparaginase was discontinued in four patients due to adverse events (AEs), including two patients with allergy to L-asparaginase (both in the second course), one patient with pancreatitis (grade 2, in the first course), and one patient with liver function derangement (in the first course). In two of these four patients, L-asparaginase was readministered at a 50% dose reduction. One allergic patient received simultaneously prednisolone 1 mg/kg. In another four patients, L-asparaginase was also stopped per protocol, owing to AEs of preceding agents (methotrexate, ifosfamide, and etoposide), including two patients with infections and two patients with thrombocytopenia. The relative dose-intensity of L-asparaginase in the first course of SMILE was 81%. Two of these eight patients who had L-asparaginase discontinued achieved CR. The relative dose-intensity of CR patients was 92%.

Additional courses of SMILE were given for 21 patients (one course, 10 patients; two courses, three patients; three courses, two patients; four courses, six patients). The median number of courses of SMILE administered was three (range, one to six courses). Treatment of the 28 patients who completed two courses of SMILE were as follows: chemotherapy only (n = 7), autologous HSCT (n = 4), or allogeneic HSCT (n = 17; myeloablative, n = 15, nonmyeloablative, n = 2). No difficulties in mobilizing peripheral blood HSC were encountered in the four patients who received autologous HSCT. Among the seven patients who did not complete the protocol treatment, two of them received no additional treatment and died as a

Table 3. Incidence and Maximum Severity of Adverse Events (N = 38)

Adverse Event	Grade 3 No.	Grade 3 %	Grade 4 No.	Grade 4 %
Hematologic				
Leukopenia	9	24	29	76
Neutropenia	3	8	35	92
Anemia	18	47	1	3
Thrombocytopenia	9	24	15	40
Nonhematologic				
Hypofibrinogenemia	4	11	0	0
APTT elongation	4	11	0	0
Hypoalbuminemia	6	16	0	0
Hyperbilirubinemia	3	8	1	3
AST elevation	12	32	0	0
ALT elevation	10	26	2	6
Creatinine	2	5	0	0
Hyponatremia	11	29	1*	3
Hyperglycemia	7	18	0	0
Amylase	6	16	1*	3
Appetite loss	8	21	1*	3
Diarrhea	4	11	0	0
Nausea	5	13	0	0
Mucositis	5	13	0	0
Vomiting	2	5	0	0
Infection	17	45	6†	16
Somnolence	1	3	2	5
Encephalopathy	0	0	1	3

NOTE. Grade 3 hypernatremia, allergic reaction, fever, and dehydration were observed in one patient each.
Abbreviation: APTT, activated partial thromboplastin time.
*Related to grade 2 pancreatitis in one patient.
†Including the two patients who died as a result of infection (two treatment-related deaths).

Table 4. Response After Two Cycles of SMILE Chemotherapy (N = 38)

Response	All Patients (N = 38) No.	All Patients (N = 38) %	Newly Diagnosed Stage IV (n = 20) No.	Newly Diagnosed Stage IV (n = 20) %	First Relapse (n = 14) No.	First Relapse (n = 14) %	Refractory to the First-Line Therapy (n = 4) No.	Refractory to the First-Line Therapy (n = 4) %
CR	17	45	8	40	9	64	0	0
PR	13	34	8	40	4	29	1	25
NR	1	3	1	5	0	0	0	0
PD	4	10	1	5	1	7	2	50
ED	3	8	2	10	0	0	1	25

Abbreviations: CR, complete response; ED, early death; NR, no response; PD, progressive disease; PR, partial response; SMILE, steroid (dexamethasone), methotrexate, ifosfamide, L-asparaginase, and etoposide.

result of disease. Three patients were treated with other chemotherapy, and two of them underwent allogeneic HSCT without response.

Toxicity

Table 3 lists all grade 3 or 4 AEs that occurred in the 38 eligible patients who were enrolled onto this trial. After the death of initial two patients from grade 5 infections (patients 1 and 2; see Appendix, online only), the protocol was revised to include a careful assessment of infection and the incorporation of a lymphocyte count of $\geq 500/\mu L$ into the eligibility criteria. There were no subsequent treatment-related deaths.

Grade 4 neutropenia was common (92%). The nonhematologic grade 4 toxicities included infection (n = 6), hyperbilirubinemia (n = 1), ALT elevation (n = 2), and encephalopathy (n = 1); two patients experienced grade 4 somnolence, which was complicated by a grade 3 infection in one patient and by grade 4 encephalopathy in the other patient. One patient experienced grade 2 pancreatitis and had complications from grade 4 hyponatremia, hyperamylasemia, and appetite loss. The most common grade 3 nonhematologic AE was infection (45%). Allergic reactions due to L-asparaginase of any grade were observed in five patients (three with grade 1, one with grade 2, and one with grade 3). The toxic profiles according to disease status at the time of study entry (newly diagnosed/relapsed/refractory) are shown in Appendix Table A1 (online only). The grade 4 hematologic toxicity rates of newly diagnosed and relapsed patients were 95% and 93%, respectively. The grade 4 nonhematologic toxicity rates of newly diagnosed and relapsed patients were 35% and 14%, respectively. None of these differences were statistically significant ($P = .99$ and $P = .25$). No clinical predictors of toxicity were found. Only hyponatremia was associated with newly diagnosed and refractory diseases.

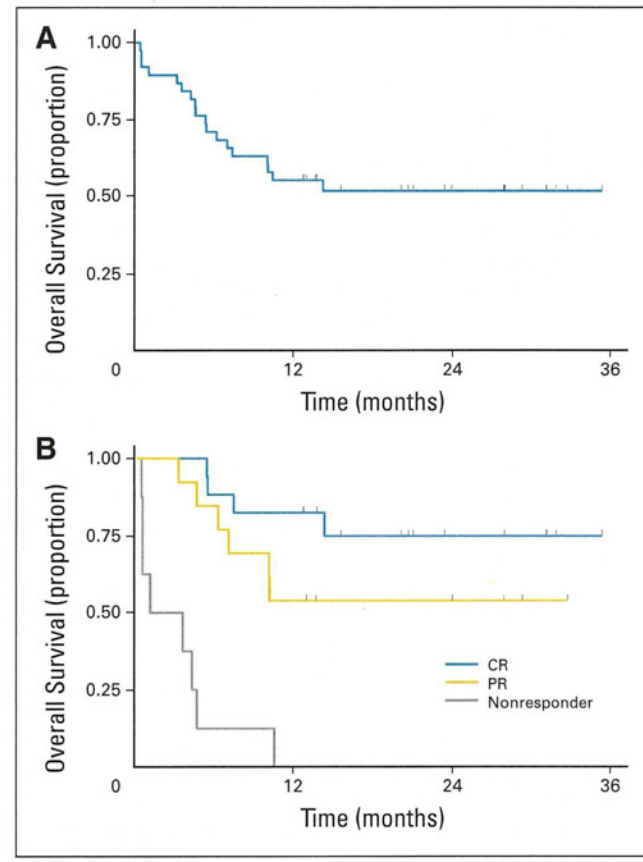

Fig 1. Kaplan-Meier estimates of overall survival (OS) of patients treated with steroid (dexamethasone), methotrexate, ifosfamide, L-asparaginase, and etoposide chemotherapy. (A) The 1-year OS of 38 patients was 55% (95% CI, 38% to 69%). The median follow-up of survivors was 24 months (range, 13 to 35 months). (B) The 1-year OS was 82% (95% CI, 55% to 94%) for patients who attained complete response (CR) and 54% (95% CI, 25% to 76%) for those who attained partial response (PR).

Efficacy and Survival

Among the 38 eligible patients, the response was CR in 17 patients (45%), PR in 13 patients, no response in one patient, progressive disease in four patients, and early death in three patients (Table 4). The ORR was 79% (90% CI, 65% to 89%). There were no differences in either the ORR or CR rate between patients with newly diagnosed stage IV disease and those with first-relapse disease. With respect to progressive disease in four patients, one occurred during the first course of SMILE, one after the first course, and two after the completion of two courses.

The median follow-up time of the living patients was 24 months, with a range of 13 to 35 months. The OS rate at 1 year, which was one of the secondary end points, was 55% (95% CI, 38% to 69%; Fig 1A). The progression-free survival (PFS) at 1 year was 53% (95% CI, 36% to 67%). The patients who attained response with SMILE chemotherapy had a higher OS (Fig 1B). The OS and PFS by the disease state at entry are shown in Figure 2A and 2B. Patients with relapsed disease showed better 1-year OS (79%) and PFS (71%) as compared with patients with refractory disease (P = .04 and .05, respectively). The survival curves of patients (excluding early deaths; n = 35) according to the type of poststudy therapy (autologous/allogeneic HSCT/chemotherapy) are shown in Figures 2C and 2D. Patients who received autologous HSCT seemed to show better OS and PFS, but the difference was not statistically significant. Univariate analysis for OS showed that presence of B symptoms (HR, 3.1, P = .01), performance status of 1 or 2 (HR, 3.1, P = .002), elevated serum lactate dehydrogenase (HR, 6.1, P = .001), and hemoglobin of less than 12 g/dL (HR, 3.9, P = .007) were significant prognostic factors.

DISCUSSION

Our results indicate that SMILE chemotherapy is effective for the treatment of newly diagnosed stage IV, relapsed, or refractory ENKL. The ORR after two cycles of SMILE (79%; 90% CI, 65% to 89%) clearly exceeded the threshold ORR (35%).[20] The 1-year OS rate (55%) was much improved compared with the previous treatment strategy.

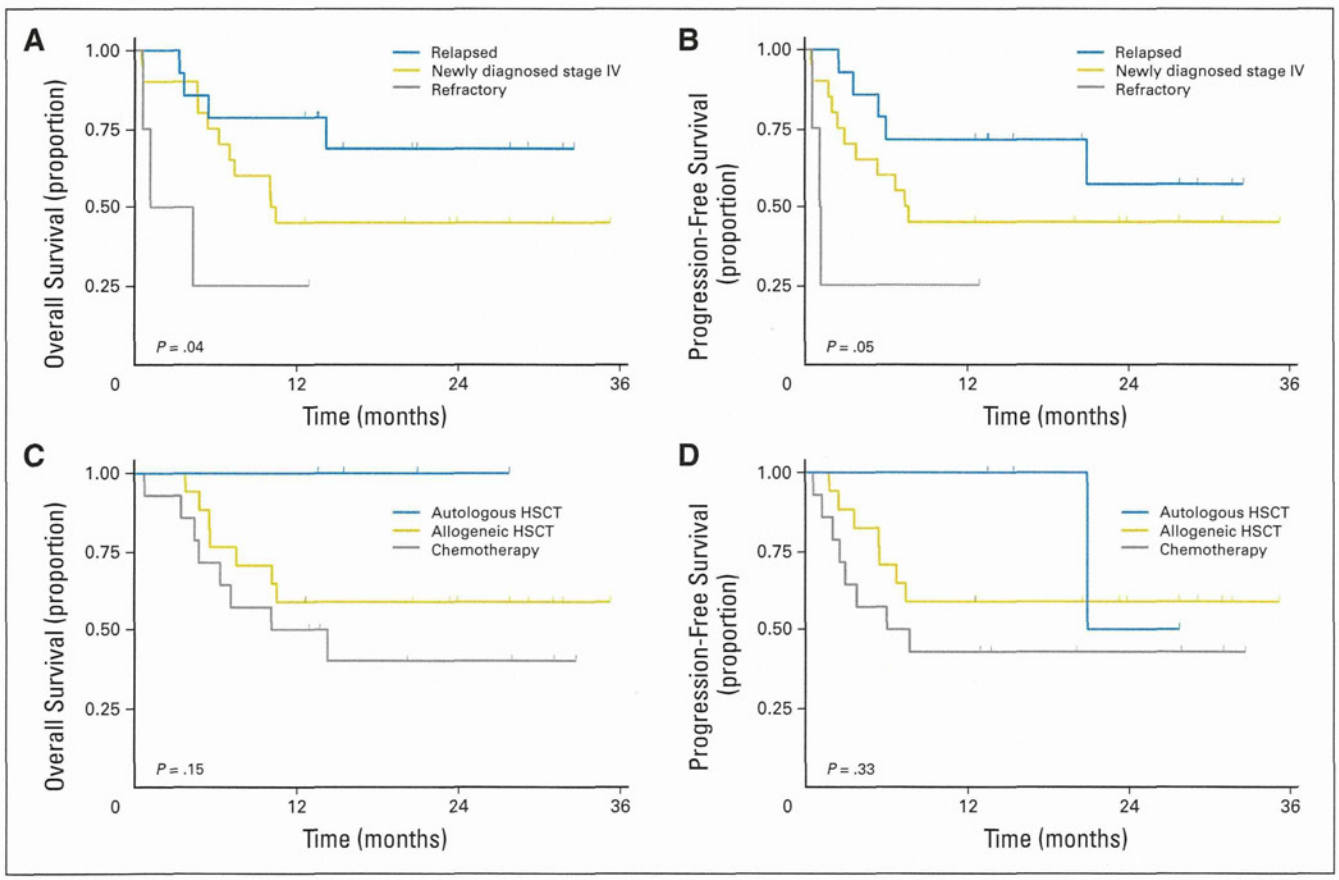

Fig 2. Kaplan-Meier estimates of overall survival (OS) and progression-free survival (PFS) of patients by the subgroup analysis. (A) OS of patients by the disease state at entry. The 1-year OS was 45% (95% CI, 23% to 65%) for patients with newly diagnosed stage IV disease, 79% (95% CI, 47% to 93%) for patients with relapsed disease, and 25% (95% CI, 1% to 67%) for patients with refractory disease. The difference was statistically significant (P = .04). (B) PFS of patients by the disease state at entry. The 1-year PFS was 45% (95% CI, 23% to 65%) for patients with newly diagnosed stage IV disease, 71% (95% CI, 41% to 88%) for patients with relapsed disease, and 25% (95% CI, 1% to 67%) for patients with refractory disease. The difference was statistically significant (P = .05). (C) OS of patients excluding early death (n = 35) by type of poststudy therapy. The 1-year OS was 100% for patients who received autologous hematopoietic stem-cell transplantation (HSCT), 59% (95% CI, 36% to 78%) for patients who received allogeneic HSCT, and 41% (95% CI, 19% to 63%) for patients treated with chemotherapy only. The difference was not statistically significant (P = .15). (D) PFS of patients excluding early death by type of poststudy therapy. The 1-year PFS was 100% for patients who received autologous HSCT, 59% (95% CI, 36% to 78%) for patients who received allogeneic HSCT, and 35% (95% CI, 14% to 57%) for patients treated with chemotherapy only. The difference was not statistically significant (P = .33).

With regard to the safety of SMILE, myelosuppression and infection should be carefully monitored during and after SMILE chemotherapy. To avoid severe AEs, the use of granulocyte colony-stimulating factor is considered mandatory, starting on day 6 and continuing until recovery beyond the nadir. In addition, full-dose administration of SMILE should be avoided for patients who are in poor condition, including those with lymphopenia less than 500/μL or large tumor burden. A lymphocyte count was added to the eligibility criteria because all three of the patients who died of neutropenic infection in the phase I and phase II SMILE studies had low lymphocyte counts before treatment. Decreased-dose SMILE[21] and less-intensive L-asparaginase chemotherapies[22-24] are candidate strategies for those patients with poor pretreatment conditions.

L-asparaginase–based chemotherapy has been highlighted as a promising treatment for ENKL. L-asparaginase was shown to induce apoptosis of ENKL cells in vitro; this result was attributed to low asparagine synthetase expression.[25] In fact, there were several case reports in the early 2000s in which ENKL showed an excellent response to L-asparaginase.[26-30] Recently, a phase II study of L-asparaginase, methotrexate, and dexamethasone (AspaMetDex) for relapsed or refractory ENKL was reported by a French group.[22] Nineteen patients were enrolled, and the CR rate was 61%. The median survival time was 12.2 months, and the 1-year OS was 45%. The AspaMetDex therapy is also promising, but there are several differences from the SMILE study. First, 53% of patients in our SMILE study had newly diagnosed stage IV ENKL which showed poor prognosis with conventional chemotherapy.[6] In contrast, the GELA (Groupe d'Etude des Lymphomes de l'Adulte)/GOELAMS (Groupe Ouest-Est des Leucémies et des Autres Maladies du Sang) study included only patients with relapsed/refractory disease. This resulted in a different ratio of patients with advanced-stage disease between the SMILE study (27 of 38 patients, 71%) and the AspaMetDex study (seven of 19 patients, 37%). Second, 17 of the 19 patients were initially treated with anthracycline-based chemotherapy in the French study. In contrast, 81% of the patients who had prior therapy in our study received platinum-based chemotherapy before SMILE, which suggests that different patient groups were selected in the two studies. Currently, these SMILE and AspaMetDex regimens are both promising for relapsed/refractory ENKL. A comparative study is required for a conclusion, but is not realistic for this type of rare lymphoma.

The optimal course of SMILE chemotherapy and the most appropriate timing of HSCT for patients after two courses of SMILE remain undetermined. In addition, the optimal treatment strategy for patients who cannot undergo HSCT needs further clinical evaluation. It has been speculated that the SMILE regimen may also be effective for T-cell lymphomas because ENKL and mature T-cell lymphomas share several clinical and pathologic features, such as extranodal predilection and expression of cytotoxic molecules. This speculation should be confirmed in further clinical studies.

In conclusion, the results of this phase II study demonstrate that two cycles of SMILE is an effective chemotherapy regimen for patients with newly diagnosed stage IV, relapsed, or refractory ENKL. However, the SMILE regimen is potentially toxic, and careful patient monitoring is needed.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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Escape of leukemia blasts from HLA-specific CTL pressure in a recipient of HLA one locus-mismatched bone marrow transplantation

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ABSTRACT

A case of leukemia escape from an HLA-specific cytotoxic T lymphocyte (CTL) response in a recipient of bone marrow transplantation is presented. Only the expression of HLA-B51, which was a mismatched HLA locus in the graft-versus-host direction, was down-regulated in post-transplant leukemia blasts compared with that in pre-transplant blasts. All CTL clones, that were isolated from the recipient's blood when acute graft-versus-host disease developed, recognized the mismatched B*51:01 molecule in a peptide-dependent manner. The pre-transplant leukemia blasts were lysed by CTL clones, whereas the post-transplant leukemia blasts were not lysed by any CTL clones. The IFN-γ ELISPOT assay revealed that B*51:01-reactive T lymphocytes accounted for the majority of the total alloreactive T lymphocytes in the blood just before leukemia relapse. These data suggest that immune escape of leukemia blasts from CTL pressure toward a certain HLA molecule can lead to clinical relapse after bone marrow transplantation.

1. Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is curative for leukemia by virtue of the immune reaction mediated by donor T lymphocytes, termed the graft-versus-leukemia (GVL) effect [1]. For HSCT recipients from HLA-matched donors, the GVL effect can be triggered by minor histocompatibility antigens [2–4], and several studies using sequential flow cytometric analysis with tetramers have clearly demonstrated that minor histocompatibility antigen-specific T lymphocytes increase in frequency in the recipient's blood before and during clinical regression of leukemia [5–10]. On the other hand, for HLA-mismatched HSCT recipients, extremely limited biological studies have demonstrated that the GVL effect can be mediated by mismatched HLA-specific donor T lymphocytes [11].

Allogeneic HSCT is a well-established immunotherapy for leukemia, but, unfortunately, some recipients relapse after transplantation. It is difficult to evaluate the role of individual factors in relapse. Nevertheless, it is reasonable to assume that the selective pressure exerted by donor T lymphocytes can lead to the outgrowth of pre-existing leukemia variants that have lost expression of gene products such as HLA molecules. Some studies have demonstrated loss of the mismatched HLA haplotype in the leukemia blasts of HSCT recipients as a consequence of loss of heterozygosity in chromosome 6 [12–14]. However, the mechanisms involved in leukemia relapse after HLA locus-mismatched HSCT remain largely uninvestigated.

This paper presents a case of selective HLA down-regulation in post-transplant leukemia blasts but not in pre-transplant blasts of a recipient who received bone marrow transplantation from an HLA one locus-mismatched donor. All cytotoxic T lymphocyte (CTL) clones that were isolated from the recipient's blood during acute graft-versus-host disease (GVHD) demonstrated cytotoxicity specific for the mismatched HLA-B molecule, lysed pre-transplant blasts but not post-transplant blasts, and persisted in the patient's blood until leukemia relapse. These results suggest that immune escape of leukemia blasts from CTL pressure toward a certain HLA allele can lead to clinical relapse.

2. Patient, materials and methods

2.1. Patient

A 24-year-old man with primary refractory T lymphoblastic leukemia/lymphoma received allogeneic bone marrow transplantation without ex vivo T lymphocyte depletion from his mother. Because the patient had neither a sibling nor an HLA-matched unrelated donor, his mother was chosen as an alternative donor. PCR sequencing-based typing for HLA alleles of the patient and mother revealed one HLA-B allele mismatch in

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Table 1
HLA types of the patient and donor.

	A	B	C	DRB1	DQB1	DPB1
Patient	1101/2402	5401/5101	0102/–	0901/–	0303/–	0501/–
Donor	1101/2402	5401/5201	0102/1202	0901/1502	0303/0601	0501/–

The mismatched HLA allele in the graft-versus-host direction is underlined.

the graft-versus-host direction (Table 1). The preparative regimen consisted of 180 mg/m^2 melphalan and 12 Gy total body irradiation. GVHD prophylaxis consisted of 0.03 mg/kg tacrolimus and short-term methotrexate. Neutrophil engraftment (neutrophil count $\geqslant 0.5 \times 10^9$/l) was achieved 14 days after transplantation with full donor-type chimera. The patient developed severe acute GVHD involving the skin, gut, and liver on day 46 (maximum stage: skin 3, gut 2, and liver 1; maximum grade: III on day 53), evaluated according to previously published criteria [15]. Acute GVHD was temporarily controlled by additional immunosuppressants, but it was incurable and transitioned to chronic GVHD. On day 261, the patient relapsed with ascites, a hydrocele, and a subpapillary tumor. Leukemia blasts in the ascites fluid were confirmed by cytological examination. Immunosuppressant therapy was required to control GVHD until his death on day 279.

2.2. Cell culture

CTL clones were isolated from a blood sample as described previously [16]. Briefly, peripheral blood mononuclear cells (PBMCs) obtained from the recipient on day 56, when severe acute GVHD developed, were stimulated in vitro with aliquots of γ-irradiated PBMCs that had been obtained from the recipient pre-transplant and cryopreserved. After three weekly stimulations, the CTL clones were isolated from the polyclonal T lymphocyte culture by limiting dilution. The CTLs were expanded by stimulation every 14 days with 30 ng/ml OKT3 monoclonal antibody (Janssen Pharmaceutical), using unrelated allogeneic γ-irradiated (25 Gy) PBMCs and γ-irradiated (75 Gy) EB virus-transformed lymphoblastoid cells (B-LCL) as feeder cells. The culture medium consisted of RPMI-1640-HEPES (Sigma–Aldrich) containing 10% pooled, heat-inactivated human serum, and recombinant human IL-2 (R&D Systems). The T lymphocytes were used in assays 14 days after stimulation or 1 day after thawing of a frozen aliquot. All samples were collected after written informed consent had been obtained. B-LCLs were maintained in RPMI-1640-HEPES with 10% FBS. COS cells were maintained in DMEM (Sigma–Aldrich) with 10% FBS.

2.3. Flow cytometric analysis

Leukemia blasts were incubated at 37 °C for 30 min with anti-HLA-A24/A23 (One lambda), anti-HLA-A11/A1/A26 (One lambda), and anti-HLA-B51/B52/B49/B56 (One lambda) antibodies to detect A24, A11, and B51, respectively, of patient cells followed by incubation at 37 °C for 15 min with fluorescein isothiocyanate-conjugated antimouse IgM (Beckman Coulter). To detect HLA-DR9 of patient cells, leukemia blasts were incubated at 37 °C for 30 min with fluorescein isothiocyanate-conjugated anti-HLA-DR antibody (BD Pharmingen). Antibody to detect HLA-B54 without cross-reaction to B51 was not available. After washing, the cells were analyzed by a BD FACSAria (BD Biosciences). Leukemia blasts were sorted by BD FACSAria with anti-CD7 (BD Biosciences) and anti-CD10 (eBiosciences) antibodies from pre-transplant bone marrow and post-transplant ascites fluid samples. The purities of pre-transplant and post-transplant blasts were ~62% and ~99%, respectively. CTL clones were analyzed using three-color flow cytometry for expression of CD3, CD4, and CD8 using phycoerythrin-cyanin 5.1-conjugated anti-CD3 (Beckman Coulter), phycoerythrin-conjugated anti-CD4 (BD Biosciences), and fluorescein isothiocyanate-conjugated anti-CD8 (BD Biosciences) antibodies.

2.4. Chromium release assay

Leukemia blasts and B-LCLs were used as target cells in a cytotoxicity assay. Leukemia blasts and B-LCLs were labeled for 2 h with ^{51}Cr. After washing, the cells were dispensed at 2×10^3 cells/well into triplicate cultures in 96-well plates and incubated for 4 h at 37 °C with CTL clones at various E:T ratios. Percent-specific lysis was calculated as [(experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm)] ×100.

2.5. Determination of T cell receptor (TCR)-Vβ gene usage and nucleotide sequences

TCR Vβ usage was assessed by RT-PCR using primers covering the entire families of functional TCR Vβ chains [17–19]. Briefly, total RNA was extracted from individual CTL clones, and cDNA was synthesized using SuperScript III RT (Invitrogen). RT-PCR reactions were carried out with the appropriate Vβ sense primers specific for different Vβ families and a primer specific for the constant region of TCR-β. Subsequently, the complementarity determining region 3(CDR3) of each positive PCR product was sequenced with corresponding antisense primer. TCR Vβ gene usage was determined by the international ImMunoGeneTics information system (IMGT) software, IMGT/V-QUEST (http://www.imgt.org/).

2.6. HLA-B cDNA constructs

Total RNA was extracted from the patient and donor B-LCLs and converted into cDNA. Constructs containing the full-length *HLA-B*51:01*, *B*52:01*, and *B*54:01* cDNA were generated from the cDNA by PCR and cloned into the pEAK10 expression vector (Edge BioSystems). Two mutated *HLA-B*51:01* cDNA constructs, in which amino acid at position 63 or 67 was substituted with the corresponding amino acid in *B*52:01*, and two more mutated *HLA-B*51:01* cDNA constructs, in which the amino acid at position 194 or 199 was substituted with the corresponding amino acid in *B*44:03*, were produced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene).

2.7. Transfection of B-LCLs and COS cells with HLA cDNA

B-LCL (5×10^6) were transfected by electroporation (200 V, 500 μFD) in 200 μl of potassium-PBS with the 15 μg of pEAK10 plasmid encoding *HLA-B*51:01* cDNA and selected with puromycin (Edge BioSystems), beginning 48 h after transfection. Three days after selection, they were used as targets in a chromium release assay. COS cells (5×10^3) were plated in individual wells of 96-well flat-bottom plates and transfected with 100 ng of the pEAK10 plasmid encoding *HLA-B*51:01*, *HLA-B*52:01*, *HLA-B*54:01*, or mutated *HLA-B*51:01* cDNA using the FuGENE 6 Transfection Reagent (Roche).

2.8. CTL stimulation assay

COS transfectants (5×10^3) were cocultured with CTL clones (2×10^4) in individual wells of 96-well flat-bottom plates for 24 h at 37 °C, and IFN-γ production was measured in the supernatant using ELISA (Endogen).

2.9. Enzyme-linked immunospot (ELISPOT) assay

T lymphocytes were isolated from recipient's PBMCs by negative depletion using the Pan T Cell Isolation Kit II (Miltenyi Biotec) and used as responder T cells. Responder T cells at a concentration of 2×10^5 per well were plated in individual wells of the 96-well MultiScreen-IP filter plates (Millipore) coated with anti-human interferon (IFN)-γ antibody (5 μg/ml; Mabtech) and tested in triplicate against a total of 2×10^5 stimulator cells: patient B-LCL, donor B-LCL, and *HLA-B*51:01*-transfected donor B-LCL. The plates were incubated for 24 h at 37°C, washed, and incubated with biotinylated anti-human IFN-γ antibody (1 μg/ml; Mabtech) for 2 h at room temperature. After addition of streptavidin (Fitzgerald Industries International) to the wells, the plates were developed with a 3-amino-9-ethylcarbazol substrate kit (Vector Laboratories). Spots were counted using a microscope, and mean numbers were calculated from triplicate wells after subtraction of the number of spots obtained with medium alone.

3. Results

3.1. Selective down-regulation of HLA-B locus in post-transplant leukemia blasts

To determine whether expressions of some HLA loci in post-transplant relapsed leukemia blasts were down-regulated or lost, flow cytometric analysis was performed for HLA-A*24:02, A*11:01, B*51:01, and DR*09:01 using anti-HLA-A24/A23, -HLA-A11/A1/A26, -HLA-B51/B52/B49/B56, and -pan HLA-DR antibodies, respectively. The expression of B*51:01 was down-regulated in post-transplant leukemia blasts compared with that in pre-transplant blasts, whereas expressions of A*24:02, A*11:01, and DR*09:01 were the same or higher in post-transplant blasts than in pre-transplant blasts (Fig. 1). These data led us to question whether B*51:01-selective pressure mediated by donor T lymphocytes was present in the patient post-transplant.

3.2. Isolation of alloreactive CTL clones

Ten CTL clones, termed TK1 to TK10, were isolated from the peripheral blood of the recipient during acute GVHD. In a cytotoxicity assay, all isolated clones lysed recipient B-LCL but failed to lyse donor B-LCL (Fig. 2), demonstrating that all clones were alloreactive. Flow cytometric analysis revealed that all CTL clones

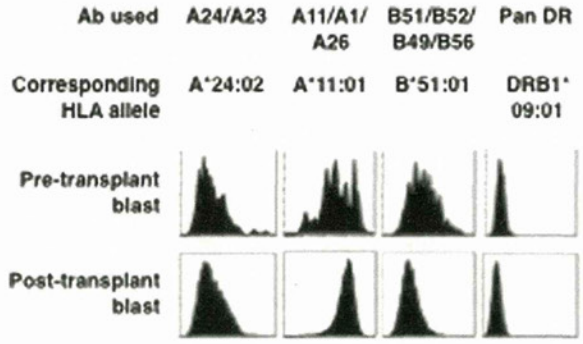

Fig. 1. HLA expression on leukemia blasts. Pre-transplant and post-transplant leukemia blasts were stained with anti-HLA-A24/A23, anti-HLA-A11/A1/A26, anti-HLA-B51/B52/B49/B56, and anti-HLA-pan DR antibodies to detect A*24:02, A*11:01, B*51:01, and DRB1*09:01, respectively. Data are representative of four experiments.

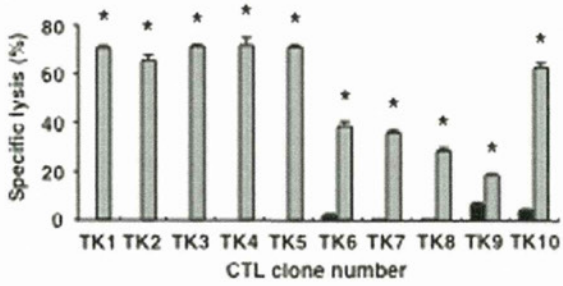

Fig. 2. Cytotoxicities of CTL clones against B-LCLs. B-LCLs that originated from the recipient (gray) and the donor (black) were used as targets for CTL clones. Specific lysis is shown as the mean and SD of triplicate cultures at an E:T ratio of 10:1. *Significant difference ($p < 0.0001$: Student's t-test) in the lysis of recipient B-LCL compared with donor B-LCL. Data are representative of three experiments.

Table 2
Clonotypes of isolated CTL clones.

CTL	TCR Vβ	Nucleotide and deduced amino acid sequences of complementarity determining region 3																		
TK1	Vβ6.5	GCC	AGC	AGT	CCC	GGG	ACT	AGC	GGA	ACC	TAC	GAG	CAG	TAC	TTC					
		A	S	S	P	G	T	S	G	T	Y	E	Q	Y	F					
TK2	Vβ20	AGT	CAG	GGG	CCG	GCG	GTT	ACC	GGG	GAG	CTG	TTT	TTT							
		S	Q	G	P	A	V	T	G	E	L	F	F							
TK3	Vβ20	AGT	CAG	GGG	CCG	GCG	GTT	ACC	GGG	GAG	CTG	TTT	TTT							
		S	Q	G	P	A	V	T	G	E	L	F	F							
TK4	Vβ19*1	GCC	AGT	ACT	TGG	GGT	TAC	CCA	CAG	GGG	CCC	GGT	GCG	GAT	ACC	GGG	GAG	CTG	TTT	TTT
		A	S	T	W	G	Y	P	Q	G	P	G	A	D	T	G	E	L	F	F
TK5	Vβ19*1	GCC	AGT	ACT	TGG	GGT	TAC	CCA	CAG	GGG	CCC	GGT	GCG	GAT	ACC	GGG	GAG	CTG	TTT	TTT
		A	S	T	W	G	Y	P	Q	G	P	G	A	D	T	G	E	L	F	F
TK6	Vβ12	GCC	AGC	AGT	TTA	GCT	AGC	GGG	AGG	GCC	TCC	CAT	GAG	CAG	TTC	TTC				
		A	S	S	L	A	S	G	R	A	S	H	E	Q	F	F				
TK7	Vβ12	GCC	AGC	AGT	TTA	GCT	AGC	GGG	AGG	GCC	TCC	CAT	GAG	CAG	TTC	TTC				
		A	S	S	L	A	S	G	R	A	S	H	E	Q	F	F				
TK8	ND																			
TK9	Vβ12	GCC	AGC	AGT	TTA	GCT	AGC	GGG	AGG	GCC	TCC	CAT	GAG	CAG	TTC	TTC				
		A	S	S	L	A	S	G	R	A	S	H	E	Q	F	F				
TK10	Vβ2	GCC	AGC	AGT	GAC	TCT	ATC	GCG	GAT	GAG	CAG	TTC	TTC							
		A	S	S	D	S	I	A	D	E	Q	F	F							

ND, not detected.