Table 4 Characteristics of patients who developed high-grade CMV antigenemia

UPN	*Sex/age*	*Diagnosis*	*Preparative regimen*	*aGVHD grade*	*Steroid at start of GCV (mg)*	*Day 0 to 1st HG-CMV (days)*	*Duration of steroid before HG-CMV (days)*	*Peak value of CMV-Ag*	*Duration of GCV/FOS (days)*	*Negative conversion*	*Second HG-CMV*	*Time from first/second HG-CMV to disease (days)*	*CMV disease*	*Survival (months)*	*Cause of death*
1	M/25	ALL	TBI	–	PSL 30	66	18	115	40/–	+	–	–	–	7.8	Relapse
2	F/40	ALL	TBI	III	mPSL 50	40	6	65	22/–	+	–	–	–	43.0+	Alive
3	M/26	CML	TBI	IV	mPSL 200	49	32	298	20/15	+	+	43/3	Pneumonia, Retinitis	38.8+	Alive
4	M/31	CML	non-TBI	IV	PSL 70	45	7	70	21/23	+	–	57	Pneumonia	3.4	TMA
5	M/44	MDS	TBI	II	PSL 15	39	13	419	9/18	+	–	–	–	33.3+	Alive
6	M/57	CML	TBI	II	mPSL 120	36	17	66	22/–	+	–	–	–	28.5+	Alive
7	M/32	MDS	TBI	III	mPSL 35	216	180	50	15/–	+	+	37/2	Pneumonia, Colitis	8.3	IA
8	M/44	NHL	TBI	I	PSL 34	637	557	157	16/–	–	–	–	–	21.6	SIRS
9	M/35	AML	TBI	II	mPSL 90	40	17	56	23/–	+	–	–	–	24.6+	Alive
10	F/25	CML	TBI	III	mPSL 250	42	19	52	11/9	+	–	227	Gastritis	21.5+	Alive
11	F/38	AML	TBI	II	mPSL 40	53	14	95	23/–	+	+	303/101	Colitis	16.9+	Alive
12	M/28	NHL	TBI	II	mPSL 30	48	24	83	20/–	+	+	–	–	9.7	Relapse
13	F/33	AA	ATG	–	–	94	–	338	27/–	–	–	–	–	3.8+	Alive
14	F/60	AML	FB 16	III	mPSL 90	43	9	821	72/–	+	–	0	Colitis	3.8	Relapse
15	M/49	Pancreatic ca.	Gem+FB	III	mPSL 80	59	13	251	22/–	+	+	–	–	6.1+	Alive
16	M/56	NHL	non-TBI	II	PSL 25	157	15	186	35/–	–	–	12	Colitis	6.4+	Alive
17	M/33	AML	TBI	III	PSL 60	269	224	86	47/–	+	–	–	–	26.9+	Alive

UPN = unique patient number; aGVHD = acute graft-versus-host-disease; HG-CMV = high-grade cytomegalovirus; GCV = ganciclovir; FOS = foscarnet; AA = aplastic anemia; ALL = acute lymphoblastic leukemia; AML = acute myelogenous leukemia; CML = chronic myelogenous leukemia; MDS = myelodysplastic syndrome; NHL = non-Hodgkin' lymphoma; ATL = adult T-cell leukemia/lymphoma; TBI = total body irradiation; Campath = alemtuzumab; ATG = antithymocyte globulin; FB = fludarabine and busulfan; Gem = gemcitabine; PSL = predonisolone; mPSL = methylpredonisolone; TMA = thrombotic microangiopathy; IA = invasive aspergillosis; SIRS = systemic inflammatory response syndrome.

those who did not (49.5 *vs* 4%, $P<0.001$, Figure 1). However, overall survival was equivalent between the two groups (59.5 *vs* 59.4% at 5 years, $P=0.79$, Figure 2). The direct causes of death in HG-antigenemia patients did not include CMV disease, but did include relapse in three, and thrombotic microangiopathy (TMA), invasive aspergillosis (IA), and systemic inflammatory response syndrome in one each.

Clinical outcome of patients who did not develop HG-antigenemia

Two of the 57 patients without HG-antigenemia developed CMV disease during GCV treatment. One developed CMV retinitis with a maximal antigenemia level of eight positive cells, who died of acute respiratory distress syndrome of unknown cause. Another developed CMV colitis with a maximal antigenemia level of 31 positive cells, who eventually died of fungal and bacterial pneumonia. The direct causes of death in 41 patients without HG-antigenemia included relapse in 15, infection in nine (two bacterial, five fungal, and two viral), noninfectious pulmonary complications in six, acute GVHD in five, multiple organ failure in three, cardiac complications in two, and TMA in one.

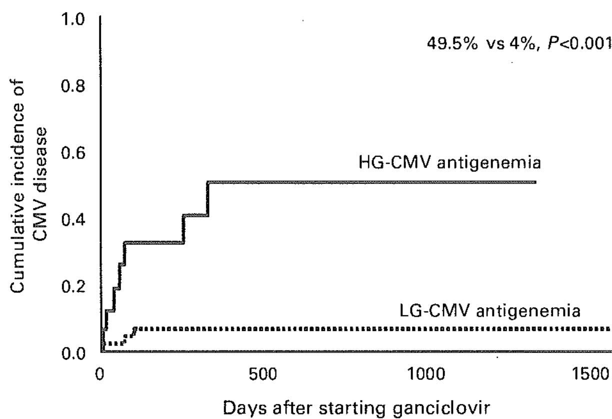

Figure 1 Cumulative incidence of CMV disease in patients who received preemptive administration of ganciclovir, grouped according to the antigenemia level. HG = high grade, LG = low grade.

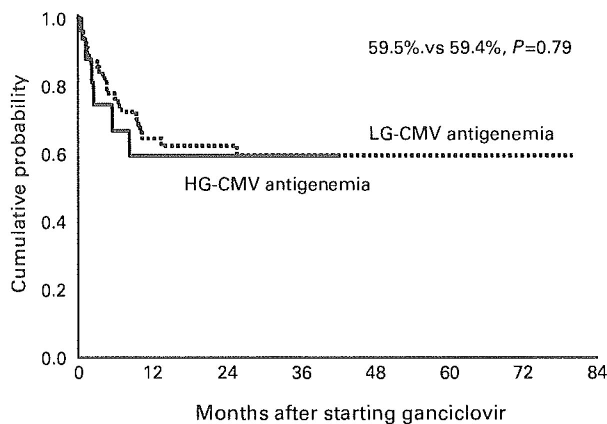

Figure 2 Overall survival of patients who received preemptive administration of ganciclovir grouped according to the antigenemia level.

Discussion

The use of systemic corticosteroids at ⩾0.5 mg/kg/day at the initiation of GCV was identified as an independent risk factor for HG-antigenemia. Although the cumulative incidence of CMV disease in patients with HG-antigenemia was significantly higher than that of patients with LG-antigenemia, overall survival was almost equivalent. We used a different threshold of CMV antigenemia to start GCV. By the use of this risk-adapted preemptive therapy, the incidence of HG-antigenemia and CMV diseases was not significantly different between those transplanted from an HLA-matched related donor and those transplanted from an alternative donor. Therefore, this risk-adapted approach could have overcome the risk due to the use of an alternative donor.

There has been no consensus on the cutoff point of HG-antigenemia. Therefore, we added an analysis by dividing the patients with a maximal antigenemia of less than 50 positive cells per two slides into low-LG- and intermediate-LG-antigenemia groups, defined by the maximal positive cells less than 10 and maximal positive cells between 10 and 49, respectively. One of 28 patients with intermediate-LG-antigenemia and one of 29 patients with low-LG-antigenemia developed CMV disease, respectively. The incidence of CMV disease in patients with intermediate-LG-antigenemia was significantly lower than in those with HG-antigenemia (4.3 *vs* 49.5%, $P=0.0015$), where as it was equivalent between low-LG-antigenemia patients and intermediate-LG-antigenemia patients (4.3% in both groups). Therefore, the cutoff value appeared to be appropriate.

The use of corticosteroids at the initiation of GCV was identified as an independent risk factor for HG-antigenemia, which agreed with the conclusion of previous studies that host immune status is most important for the response to GCV treatment.[6,14,15] We made the greatest efforts to reduce the dose of corticosteroids for patients who developed CMV reactivation, although it was difficult in patients with severe acute GVHD. In addition, a patient who received ATG as conditioning developed HG-antigenemia without corticosteroid. It has been reported that the use of ATG in the conditioning regimen leads to delayed immune recovery and is strongly associated with CMV infections.[15,16]

None of the 17 HG-antigemenia patients directly died of CMV diseases, leading the inference that a fatal outcome could be avoided with an optimal antiviral therapy. However, two patients died of TMA and IA, which might have been indirectly caused by CMV infection and/or its treatment, since CMV infection has been identified as a risk factor for TMA and IA.[17,18]

Studies that evaluated the relationship between CMV load and CMV disease have shown conflicting results. Some studies showed a significant correlation between a

high viral load and CMV disease or transplant-related death.[7,8] However, in a recent study by Nichols *et al*,[6] the antigenemia level during antiviral therapy did not correlate with either CMV disease or survival. In this study, HG-antigenemia was associated with a higher risk of CMV disease but not with inferior survival. The studies that have shown a significant association between CMV viral load and survival did not use a preemptive approach, but rather ganciclovir or foscarnet was administered after the detection of CMV-related symptoms. Thus, this discrepancy may show that preemptive therapy helped to improve outcome of high-risk patients. A major difference between Nichols's study and ours was the duration of ganciclovir administration. They administered ganciclovir until day 100 after HSCT, whereas we stopped ganciclovir when antigenemia became negative. The optimal duration of preemptive GCV is still controversial. In this study, the median duration of preemptive administration of GCV was 22 days, which was equivalent to that in previous studies.[4,12] However, the recurrence of HG-antigenemia was observed in 30% of patients after the discontinuation of GCV. Furthermore, in two of the three patients with CMV pneumonia, it developed only a median of 2.5 days after the detection of second HG-antigenemia. These patients had been persistently receiving high-dose corticosteroids for grade III or IV acute GVHD. Therefore, it might be better to extend the duration of GCV administration for patients who develop HG-antigenemia and who are still receiving high-dose corticosteroid.

In conclusion, severe immunosuppression due to high-dose steroid increased the incidence of HG-antigenemia. HG-CMV antigenemia was associated with a significantly higher incidence of CMV disease, but had no influence on overall survival, since the progression of CMV disease to a fatal outcome could be prevented by antiviral treatment. However, the establishment of optimal preemptive therapies is needed in profoundly immunosuppressive patients who are receiving high-dose corticosteroid for severe GVHD.

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Cardiac complications after haploidentical HLA-mismatched hematopoietic stem cell transplantation using *in vivo* alemtuzumab

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Summary:

Alemtuzumab is a humanized monoclonal antibody directed against human CD52 with a strong lympholytic effect. We have performed unmanipulated hematopoietic stem cell transplantation (HSCT) from 2- or 3-locus-mismatched family donors in 14 patients using *in vivo* alemtuzumab. All achieved complete donor cell engraftment and grade III–IV acute graft-*versus*-host disease was observed in only one patient. However, eight of the 14 patients developed grade II–IV cardiac complications according to Bearman's criteria. Next, we retrospectively analyzed the records of 142 adult patients who underwent allogeneic HSCT from 1995 to 2004 to evaluate whether the use of alemtuzumab was an independent risk factor for cardiac complications. Among several factors that increased the incidence of grade II–IV cardiac complications with at least borderline significance, a multivariate analysis identified the cumulative dose of anthracyclines ($P = 0.0016$) and the use of alemtuzumab ($P = 0.0001$) as independent significant risk factors. All of the cardiac complications in the alemtuzumab group were successfully treated with diuretics and/or catecholamines. Patient selection and close monitoring of cardiac function may be important in HLA-mismatched HSCT using *in vivo* alemtuzumab.
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Keywords: cardiac complications; hematopoietic stem cell transplantation; HLA-mismatch; alemtuzumab

Alemtuzumab (Campath-1H) is a humanized IgG1 antibody that recognizes CD52, a glycoprotein antigen expressed on T, B, NK, and dendritic cells.[1] The addition of *in vivo* alemtuzumab to the conditioning regimen before HLA-matched allogeneic hematopoietic stem cell transplantation (HSCT) decreases the incidence of graft-*versus*-host disease (GVHD).[2–6] We thus conducted a study to evaluate the safety of unmanipulated HSCT from a 2- or 3-locus-mismatched family donor using alemtuzumab only *in vivo* and have shown that alemtuzumab is very effective for preventing GVHD.[7] However, eight of the 14 patients developed grade II–III cardiac complications according to Bearman's criteria.[8] We describe the clinical course of cardiac complications after HLA-mismatched HSCT using *in vivo* alemtuzumab. In addition, we report the results of retrospective analyses that evaluated whether the use of *in vivo* alemtuzumab was an independent risk factor for cardiac complications after adult allogeneic HSCT.

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Patients and methods

Transplantation procedure

The study to evaluate the safety of unmanipulated HSCT from 2- or 3-locus-mismatched family donors using *in vivo* alemtuzumab was started in March 2002 after approval by the ethics committee of the University of Tokyo Hospital. The transplantation procedure has been described in detail elsewhere.[7] Briefly, the conditioning regimen consisted of total body irradiation (TBI) at 2 Gy twice daily for 3 days and cyclophosphamide at 60 mg/kg/day for 2 days. The dose of cyclophosphamide was decreased to 20 mg/kg/day for 2 days, and etoposide at 40 mg/kg/day was added instead in a patient with impaired cardiac function due to anthracyclines. For elderly patients, a non-TBI containing regimen consisting of fludarabine at 30 mg/kg/day for 6 days and busulfan at 1 mg/kg four times daily for 4 days was used. After November 2003, we added TBI at 2 Gy twice daily on day −1 and decreased the dose of busulfan to 4 mg/kg/day for 2 days. Alemtuzumab was added to these regimens at 0.2 mg/kg/day for 6 days (days −8 to −3), following pretreatment with 1 mg/kg of methyl-prednisolone. Cryopreserved donor peripheral blood (PB) stem cells were infused on day 0 without *ex vivo* manipulation. GVHD prophylaxis was with cyclosporine A and short-term methotrexate. Cyclosporine was started on day −1 at a dose of 3 mg/kg/day by continuous infusion and the dose was adjusted to maintain a blood concentration between 250 and 350 ng/ml. Cyclosporine was changed to an oral form when it could be tolerated by the patient. Methotrexate was administered at 15 mg/m^2 on day 1, and 10 mg/m^2 on days 3, 6, and 11. For patients without acute GVHD, we

started to taper cyclosporine from day 30 by 10% per week and discontinued it on day 100.

Analyses of risk factors for cardiac complications

After we observed grade II–IV cardiac complications according to Bearman's criteria in eight of the 14 patients who underwent HLA-mismatched HSCT using *in vivo* alemtuzumab, we retrospectively analyzed the records of all adult patients who had undergone allogeneic HSCT for the first time between June 1995 and June 2004 at the University of Tokyo Hospital. In the following statistical analyses, we included 142 patients for whom standard 12-lead electrocardiogram (ECG) and ultrasound cardiography (UCG) within 3 months before transplantation were available. We routinely performed these procedures on all patients before conditioning. Among them, 14 patients underwent 2- or 3-locus-mismatched HSCT using *in vivo* alemtuzumab, whereas the remaining 128 patients underwent HLA-matched or 1-locus-mismatched HSCT using standard GVHD prophylaxis with cyclosporine or tacrolimus combined with methotrexate. Cyclophosphamide at more than 100 mg/kg was used in 107 patients and TBI was used in 95 patients. The stem cell source was bone marrow (BM) in 94 patients and PB stem cells in 48.

Cardiac complications observed within 28 days after HSCT were considered regimen-related toxicities and grouped according to Bearman's criteria.[8] Potential confounding factors considered in the statistical analyses were age, sex, status of underlying disease, previous cardiac disease, smoking, serum ferritin level, cumulative dose of anthracyclines, irradiation involving the heart, heart rate, blood pressure, QT interval, QT dispersion, left ventricular ejection fraction (LVEF) evaluated by UCG, dose of cyclophosphamide in the conditioning regimen, use of TBI, use of alemtuzumab, stem cell source (BM or PB), serological/genotypical HLA-mismatch, and donor type (related or unrelated). Details of the methods used to measure these parameters have been described previously.[9] For univariate analyses, continuous variables in the two groups were compared using the unpaired *t*-test or the Mann–Whitney *U*-test, whereas categorical variables were compared using the Fisher's exact test. Factors associated with at least borderline significance ($P<0.10$) on univariate analysis were subjected to multivariate analysis using logistic regression. *P*-values of less than 0.05 were considered statistically significant.

Results

HLA-mismatched HSCT using in vivo *alemtuzumab*

In total, 14 patients underwent 2- or 3-locus-mismatched HSCT using *in vivo* alemtuzumab. There were eight males and six females with a median age of 49.5 years (range 27–60). Nine patients had active disease at transplantation. Eight received a TBI-based regimen, while six received a fludarabine-based regimen. The median number of CD34+ and CD3+ cells in the graft was 5.1×10^6 cells/kg (range 4.3–7.7) and 2.5×10^8 cells/kg (range 1.0–7.1), respectively. All patients achieved donor cell engraftment and complete donor-type chimerism with a median duration to neutrophil recovery $>500/\text{mm}^3$ of 18.5 days and platelet recovery $>20\,000/\text{mm}^3$ of 18.0 days. Only two patients developed grade II–IV acute GVHD; one patient each with grade II and III, respectively. Infection-related death was observed in one patient who died of cytomegalovirus pneumonitis on day 98. Grade II–IV regimen-related toxicities according to Bearman's criteria were observed as follows: stomatitis in nine patients, renal toxicity in two, liver toxicity in one, and cardiac toxicity in eight (57.1%), mainly with congestive heart failure (Table 1). Six patients developed grade II cardiac toxicity diagnosed by an increased cardiothoracic ratio, which was detected by routine X-ray and required the use of diuretics. Grade III cardiac toxicities were observed in two patients who responded poorly to diuretics and required catecholamines. One of them showed markedly decreased left ventricular function (EF of 24%) on UCG. Another patient had paroxysmal supraventricular tachycardia. Treatment with catecholamines resolved the symptoms in both patients. There were no long-term sequelae except that one patient with grade III cardiac toxicity showed persistent LV dysfunction on UCG.

Retrospective analyses to identify risk factors for cardiac complications

Of the 142 patients included in the retrospective analysis, 23 (16.2%) and 10 (7.0%) patients developed grade II–IV and III–IV cardiac complications, respectively, within 28 days after transplantation. The median onset of cardiac complication was 13.5 and 4.5 days after HSCT in patients who received alemtuzumab and those who did not, respectively ($P=0.02$; Figure 1). Seven died of cardiac causes a median of 3 days after the onset of cardiac complications, but all the cardiac complications in the alemtuzumab group were successfully treated with diuretics and/or catecholamines.

Univariate analyses to evaluate the impact of possible confounding factors on the incidence of grade II–IV cardiac complications identified eight factors with a *P*-value less than 0.10: smoking history ($P=0.036$), serum ferritin level ($P=0.033$), cumulative dose of anthracyclines ($P=0.001$), heart rate ($P=0.084$), EF ($P=0.070$), serological HLA-mismatch ($P=0.054$), genetic HLA-mismatch ($P=0.087$), and the use of alemtuzumab ($P=0.0002$) (Table 2a).

Among these, only the cumulative dose of anthracyclines (odds ratio 1.003, 95% confidence interval (CI) 1.001–1.005, $P=0.0016$) and the use of alemtuzumab (OR 12.1, 95% CI 3.3–44.1, $P=0.001$) were identified as independent significant risk factors on multivariate analysis (Table 2b).

Discussion

Generally, cardiac complications are uncommon after treatment with alemtuzumab.[10–13] However, a high incidence of cardiac complications was reported in four of

Table 1 Cardiac complications in patients who underwent HLA 2- or 3-locus-mismatched hematopoietic stem cell transplantation using *in vivo* alemtuzumab

(a)

No.	*Age/sex*	*Disease*	*Anthracycline dose (mg/m²)*	*TBI (Gy)*	*Engraftment (day)*	*Onset (day)*	*Bearman grade*	*Treatment*	*Outcome*
1	44/F	Ph+ALL CR1	90	12	17	10	II, Mild CHF	Diuretics	Resolved
2	27/F	ALL CR2	794	12	16	20	II, Mild CHF	Diuretics	Resolved
3	56/F	Ph+ALL NR	491	(—)	20	13	II, Mild CHF	Diuretics	Resolved
4	45/M	AML PIF	186	12	18	14	II, Mild CHF	Diuretics	Resolved
5	41/F	ALL CR1	310	12	29	15	III, Severe CHF & arrhythmia	Diuretics & catecholamines	Resolved
6	57/M	AML PIF	140	4	20	14	II, Mild CHF	Diuretics	Resolved
7	54/M	MDS NR	0	4	12	6	II, Mild CHF	Diuretics	Resolved
8	33/F	AML NR	587	12	43	8	III, Severe CHF	Diuretics & catecholamines	Resolved

(b)

No.	*CTR before HSCT (%)*	*CTR at onset (%)*	*EF before HSCT (%)*	*EF at onset (%)*	*BW before HSCT (kg)*	*BW at onset (kg)*	*Oximetry at onset (%)*	*ECG findings other than sinus tachycardia*
1	52.4	56.0	66	—	61.1	62.0	98	—
2	43.3	47.8	56	47	46.4	48.8	98	—
3	46.0	54.1	70	—	45.6	48.0	97	—
4	39.8	48.3	58	—	65.6	68.7	96	—
5	43.0	51.4	63	—	44.5	49.6	95	PSVT
6	49.0	55.3	55	—	56.1	58.3	92	—
7	45.1	51.0	65	—	67.3	69.2	92	—
8	41.4	57.9	55	24	55.4	60.0	96	—

TBI = total body irradiation; CR = complete remission; PIF = primary induction failure; NR = not in remission after relapse; onset = onset of cardiac complications; CHF = congestive heart failure.

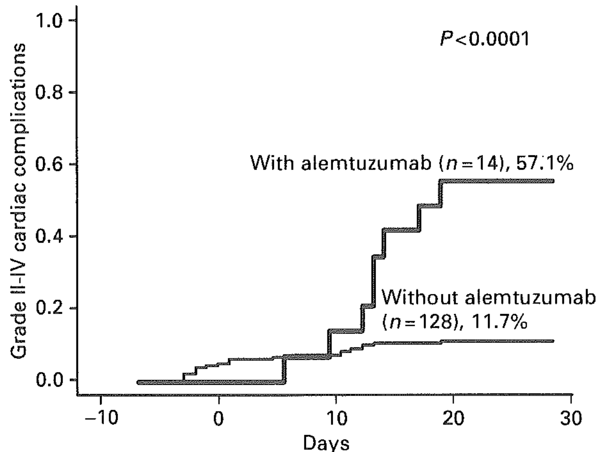

Figure 1 Cumulative incidence of grade II–IV cardiac complications according to Bearman's criteria, grouped according to the use of *in vivo* alemtuzumab in the conditioning regimen.

eight patients who received alemtuzumab for mycosis fungoides or Sézary syndrome.[14] The expression of CD52 was not observed on cardiac myocytes and, thus, cytokine-release syndrome after alemtuzumab infusion was considered to be responsible for the cardiac complications,[14] because inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1 (IL-1) and interleukin-6 (IL-6) have been reported to be responsible for the development and progression of heart failure.[15,16]

The frequent cardiac complications in our HLA-mismatched HSCT study could not be explained solely by alemtuzumab, since cardiac complications were infrequently mentioned in HLA-matched HSCT with reduced-intensity conditioning.[2–6] Therefore, cardiac complications in our series might have resulted not only from alemtuzumab but also from the intensive conditioning and/or increased cytokine secretion associated with the engraftment of HLA-mismatched donor cells. In fact, the median duration to cardiac complications after HSCT was significantly longer in patients who received alemtuzumab and most of the cardiac complications after allogeneic HSCT using alemtuzumab were observed in the peri-engraftment period (Table 1), when the secretion of various cytokines is known to increase.[17]

We monitored cardiac function after HSCT by daily measurements of body weight, pulse oximeter oxygen saturation and weekly chest X-ray. Thus, we were able largely to avoid fatal cardiac complications after HSCT using alemtuzumab. However, two patients developed grade III cardiac complications that required catecholamine support. An evaluation of the value of possibly more sensitive markers, such as plasma brain natriuretic peptide level, is thus warranted.[18] The use of diuretics may be sufficient if cardiac complications can be detected at an early stage.

In conclusion, although *in vivo* alemtuzumab is very effective for preventing GVHD even in HLA-mismatched HSCT, the use of *in vivo* alemtuzumab along with myeloablative conditioning for HLA-mismatched HSCT may increase the incidence of reversible cardiac compli-

Table 2 Risk factors for grade II IV cardiac complications according to Bearman's criteria

(a) Univariate	*Grade II–IV cardiac complications*		*P-value*
	Positive (n=23)	*Negative (n=119)*	
Sex (male)	52.2%	69.7%	0.145
Age (>40 years)	39.1%	54.6%	0.254
Disease status (high)	60.9%	58.0%	0.113
History of cardiac disease	17.4%	7.6%	0.228
Smoking	21.7%	47.1%	0.036
Ferritin level (log(ferritin))	2.804	2.514	0.033
Cumulative dose of anthracyclines	350.8	175.5	0.001
History of radiation involving heart	0%	2.5%	0.999
Vital sign			
Heart rate (beats/min)	80.83	75.45	0.084
Systolic blood pressure (mmHg)	107.2	110.9	0.356
Diastolic blood pressure (mmHg)	67.7	68.5	0.720
ECG			
ECG abnormality	17.4%	13.4%	0.743
QT interval (ms)	386.3	379.9	0.447
QTc interval (ms)	444.6	424.7	0.105
QT dispersion (ms)	47.6	52.1	0.343
QTc dispersion (ms)	55.1	57.9	0.603
Echocardiography			
Echocardiographic EF (%)	61	64.6	0.070
Regimen			
Cyclophosphamide >100 mg/m^2	60.9%	78.2%	0.111
TBI	78.3%	64.7%	0.236
Alemtuzumab	34.8%	5.0%	0.0002
Stem cell			
Peripheral blood	56.5%	70.6%	0.225
Donor			
Unrelated donor	30.4%	42.0%	0.358
HLA matched (serological level)	39.1%	19.3%	0.054
HLA matched (genetic level)	47.8%	28.6%	0.087

(b) Multivariate	*Odds ratio (95% CI)*	*P-value*
Cumulative dose of anthracyclines (mg/m^2)	1.003 (1.001–1.005)	0.0016
Alemtuzumab	12.1 (3.3–44.1)	0.0001

cations. Patient selection and close monitoring of cardiac function are important during such transplants, especially in patients who have received a high cumulative dose of anthracyclines. Cardiac complications after alemtuzumab should be manageable by early detection and treatment.

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Light- and Heavy-chain Deposition Disease (LHCDD): Difficulty in Diagnosis and Treatment

Key words: light- and heavy-chain deposition disease (LHCDD), PBSCT, renal failure

Light- and heavy-chain deposition disease (LHCDD), light-chain deposition disease (LCDD), and heavy-chain deposition disease (HCDD) are rare illnesses. A comprehensive review of the clinical features, immunopathology, and molecular biology of LCDD and related disorders was provided in 1992 by Buxbaum (1) and updated by Gallo et al (2). There is little information on the prognosis of patients with LHCDD treated with autologous stem cell transplantation (ASCT).

Characteristics of LHCDD

1) Unexplained proteinuria, renal failure, hematuria, or hypertension.
2) LCDD lacks the clinical characteristics of multiple myeloma, and occurs in younger (30–50 years), predominantly female, patients.
3) A kind of monoclonal plasma cell disorder.
4) The finding of homogeneous serum and/or urinary immunoglobulin-related M protein by the agarose gel or immunofixation electrophoresis methods. LCDD-associated monoclonal Igs are predominantly kappa-type, whereas there is a predominance of lambda-type in AL amyloidosis.
5) In order to diagnose LCDD definitively, a kidney biopsy is essential. Nodular glomerulosclerosis resulting from the co-deposition of light-chains with extracellular matrix proteins is the pathologic hallmark. Skin biopsies revealed the pathologic deposits at the dermal-epidermal junction.
6) Activation of platelet-derived growth factor β (PDGF-β) and transforming growth factor β (TGF-β) may play a role in the mediating glomerurosclerosis and hyperplastic vasculopathy.
7) Major therapeutic efforts have been directed towards reducing the synthesis of M proteins. This can best be achieved by chemotherapeutic regimens that are effective in myeloma, such as melphalan-prednisone (MP), vincristine-doxorubicin-dexamethasone (VAD), and high-dose melphalan (140–200 mg/m^2) in conjunction with autologous/allogeneic hematopoietic stem cell transplantation.
8) Renal transplantation in hemodialysis patients, who showed disease stability for more than one year and no evidence of clinically significant extrarenal deposition.
9) The overall prognosis remains poor, and death eventually results from infection and failure of vital organs (by the deposition in liver, heart, and lungs) targeted by the disease process.

The case report by Sakakima et al in this issue describes a 53-year-old male with LHCDD diagnosed by the symptom of nephrotic syndrome, hypertension, Ig-G and kappa-chain deposition in glomerulus of kidney, and plasma cell dyscrasia in the bone marrow (3). The clinical course of this case was typical of LHCDD, while there was no beneficial effect on renal function with three VAD therapies, followed by early timing of autologous hematopoietic stem cell transplantation (ASCT), and there may be consideration of renal transplantation in the hemodialysis condition, because he showed disease stability of over one year and no evidence of clinically significant extrarenal deposition.

See also p 970.

The prognosis may be changed by the choice of whether performed early, as first-line therapy, or late, as rescue ASCT treatment. In a myeloma study, the treatment-related mortalities during the first posttransplant year were 9% and 14% in the early and late groups (4). Early ASCT after several conventional treatments may be recommended for the deposition-type LHCDD, amyloidosis and other plasma cell disorders. Increasing evidence indicates that high-dose melphalan followed by ASCT improves the clinical outcome of patients with LHCDD, amyloidosis (5) and other plasma cell disorders; this is especially true of patients younger than age 65. It was recently reported, however, that stem cell transplantation was not so effective in some patients. Thus, to improve the prognosis under such conditions, high-dose melphalan (140–200 mg/m^2) in conjunction with autologous/allogeneic hematopoietic stem cell transplantation needs to be done at an early date while the disease still response to the initial therapy.

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Expression of IAP-Family Proteins in Adult Acute Mixed Lineage Leukemia (AMLL)

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Inhibitor of apoptosis protein (IAP)-family proteins suppress apoptotic signaling in normal/neoplastic cells in various settings. To determine the apoptosis-resistant mechanism in adult acute mixed lineage leukemia (AMLL) with biphenotypic blasts responsible for resistance against chemotherapy, the expression levels of IAP-family proteins in AMLL bone marrow cells were analyzed by quantitative RT-PCR. The overall expression levels of IAPs were higher than those in control, AML, and ALL cells. A significant difference for the expression of survivin was observed between AMLL and AML ($P < 0.05$), and differences between AMLL and ALL were significant for the expression of survivin ($P < 0.05$), NAIP ($P < 0.05$), and XIAP ($P < 0.05$). These findings suggest that higher expression of various IAPs is associated with the chemotherapy-resistant nature of this specific type of leukemia. Am. J. Hematol. 78:173–180, 2005. © 2005 Wiley-Liss, Inc.

Key words: IAP; apoptosis; AMLL; AML; ALL; bone marrow

INTRODUCTION

The regulation of apoptotic cell death has a profound effect on the pathogenesis and progression of hematological malignancies. Acute mixed lineage leukemia (AMLL) is a relatively rare group of hematological malignancies that exhibits the expansion of biclonal or biphenotypic blasts in peripheral blood [1,2]. According to FAB criteria, AMLL may present as ALL or as one of the AML subtypes, often as M1 [2]. AMLL has a high incidence of clonal chromosomal abnormalities, the most common being the t(9;22)(q34;q11) (Ph chromosome) and structural abnormalities involving 11q23 [2]. Recently, molecular analysis revealed that the *mixed lineage leukemia* (*MLL*) gene rearrangement occurs in AMLL cases and also in a fraction of AML/ALL patients [3]. One characteristic feature of AMLL as well as *MLL* gene-rearranged leukemia is a poor patient prognosis associated with lower sensitivity to chemotherapeutic procedures [2,4]. Resistance against chemotherapy might result from the resistance to apoptosis-inducing drugs such as steroids and Ara-C [5,6]. Regarding the complicated mechanisms that regulate apoptosis in the bone marrow of acute leukemias and myelodysplastic syndromes (MDS), we previously showed that a variety of apoptosis-related molecules are active in hematopoietic cells [7–13]. However, the associated parameters and molecules involved in apoptosis in AMLL are unclear.

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IAP-family proteins, including survivin, block apoptosis induced by a variety of triggers [14,15]. Although the biochemical mechanism by which IAP-family members suppress apoptosis is under debate, survivin is known to bind directly to and inhibit caspase-3 and -7, which act as terminal effectors in apoptotic protease cascades [15,16]. The expression of survivin is ubiquitous in fetal tissues but is restricted during development and is negligible in the majority of terminally differentiated adult tissues [17,18]. However, an analysis of the differences in gene expression between normal and tumor cells reveals that survivin is a protein whose gene is most consistently overexpressed in tumor cells relative to normal tissue [19]. Survivin is prominently expressed in transformed cell lines and in many human cancers, including hematopoietic cell tumors [20]. It is also usually detected in the cytoplasm of tumor cells and is therefore widely regarded as a cytoplasmic protein [17,21,22]. However, several studies have shown the nuclear accumulation of survivin in gastric cancer cells [23] and lung cancer cells [24]. We recently reported that ALL cells principally exhibited the nuclear localization of survivin, while CLL cells exhibited cytoplasmic distribution [13]. Although the significance of this nuclear-cytoplasmic expression in tumor cells is still controversial, the subcellular localization of survivin should also be clarified for AMLL subjects.

We also reported that survivin exhibited higher levels of expression in acute lymphocytic leukemia (ALL) and that chronic lymphocytic leukemia (CLL) cases exhibited significant over-expression of survivin and cIAP2 [13]. In acute myelogenous leukemia (AML) cases, some of these IAP-family proteins, such as NAIP and XIAP, are expressed at significantly higher levels [25]. To focus on the contribution of IAPs to the expansion of blasts in AMLL, we examined cases of AMLL that exhibited biphenotypic proliferation of blasts. The expression levels of survivin tended to be high in AMLL samples compared with control bone marrow, AML, and ALL subjects. The expression of other IAPs, including cIAP1, cIAP2, NAIP and XIAP, which suppress apoptosis by inhibiting caspase and procaspase [26–29], was also observed in these samples. The significance of IAP-family proteins in resistance against chemotherapy in AMLL is discussed.

MATERIALS AND METHODS

Patients

Fresh-frozen and formalin-fixed paraffin-embedded bone marrow-aspirated samples from 13 individuals with no hematological disorders were used as normal controls (male/female 5:8; age, median 52 years, range: 25–84 years), 9 patients with AML (8 with M2 and 1 with M1 according to the FAB classification, male/female 5:3; age, median 41 years, range: 19–78 years), 7 patients with ALL (male/female 2:5; age, median 58 years, range: 46–87 years), and 8 patients with AMLL with biphenotypic blasts (male/female 4:4; age, median 50 years, range 17–73 years) were examined. To rule out the influence of aging on bone marrow cells, age-matched control cases were analyzed. Flow-cytometic analysis was routinely performed for CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD13, CD14, CD16, CD19, CD20, CD33, CD34, CD41a, CD56, and HLA-DR. Among them, the data for CD19, CD13, and CD33 were tabulated to demonstrate the biphenotypic nature of blastic cells in AMLL samples (Table I). Diagnoses were based on Catovsky's standard clinical and laboratory criteria [2] including cell morphology [30,31]. All samples were collected at the time of the initial aspiration biopsy and stored at −80°C. We selected the adult M1 or M2 AML samples and adult

TABLE I. Summary of Cases With Adult AMLL

Case no.	Age (years)	Sex	Blast (%)	Cell markers (%)			Chromosome abnormality
				CD19	CD13	CD33	
1	40	F	90.2	98.7	58.3	99.4	45.XX.der(12)t(12;22)(p13;q11) −22
2[a]	57	M	4.2	31.2	50.2	55.0	46.XY
3	67	M	94.4	95.2	67.8	0.9	36.XY.−3.−3.−5.−7.−9.−13.−15.−16.−17.−20
4	61	F	95.7	95.9	6.4	56.6	46.XX
5	17	M	96.4	99.8	53.8	50.6	46.XY
6	21	F	76.0	97.3	75.2	64.0	47.XX.+8
7	43	F	92.0	97.9	67.6	83.1	46.XX.i(8)(q10)del(9)(?q). der(9)del(9)(p22)t(9;22)(q34;q11).der(22)t(9;22)
8	73	M	69.2	89.2	45.4	13.5	46.XY.del(20)(q11.2)

[a]For case 2. material for flow-cytometric analysis was not sufficient at the time of initial diagnosis. although the diagnosis was confirmed as AMLL at the time of second biopsy. For the second biopsy sample. the blast count accounted for more than 90% of the bone marrow cells and consisted of more than 90% CD19-positive cells and more than 50% CD13/CD33-positive cells.

ALL samples for the comparison with AMLL. The AML, ALL, and AMLL samples exhibited the proliferation of blastic cells accounting for more than 80% of all bone marrow cells. The patients were not infected with viruses including HTLV-1 and had not been treated with therapeutic drugs prior to the study.

The procedures followed were in accord with the ethical standards established by the ethics committee of Tokyo Medical and Dental University.

Double Staining for Myeloid and Lymphoid Cell Markers

The phenotype of leukemic cells in AMLL was confirmed by double immunostaining using the formalin-fixed paraffin-embedded bone marrow samples. Sections were deparaffinized and incubated with monoclonal antibody against CD20 or CD79a (DAKO, Glostrup, Denmark) and polyclonal antibody against myeloperoxidase (DAKO). Next, the sections were treated with peroxidase-conjugated anti-mouse IgG followed by a DAB development system and then with alkaline phosphatase-conjugated anti-rabbit IgG (DAKO) followed by development with an alkaline phosphatase–nitroblue tetrazolium chloride–5-bromo-4-chloro-3-indolyl phosphatase development system (DAKO).

Identification of Apoptotic Cells

To identify apoptotic cells, the terminal deoxy-transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method was used as previously described [10]. Briefly, tissue sections were deparaffinized and incubated with proteinase K (prediluted, DAKO) for 15 min at room temperature. After the tissues were washed, TdT, fluorescein isothiocyanate (FITC)-dUTP and -dATP (Boehringer Mannheim, Mannheim, Germany) were applied to the sections, which were then incubated in a moist chamber for 60 min at 37°C. Anti-FITC-conjugated antibody-peroxidase (POD converter, Boehringer Mannheim) was employed to detect FITC-dUTP labeling, and color development was achieved with DAB containing 0.3% hydrogen peroxide solution. The sections were then observed under a microscope and the proportion of TUNEL-positive cells was determined by dividing the number of positively stained cells by the total cell number (count of more than 1,000 cells).

Preparation of RNA and Quantitative Assay for IAP-Family Proteins Using TaqMan RT-PCR

RNA was extracted from frozen bone marrow samples of control subjects with no hematological disorders, AML, ALL and AMLL patients using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's directions. For quantitative RT-PCR, fluorescent hybridization probes and a TaqMan PCR Core Reagents Kit with AmpliTaq Gold (PerkinElmer Cetus, Norwalk, CT) were used with an ABI Prism 7900HT Sequence Detection System (PerkinElmer, Foster City, CA). Oligonucleotides as specific primers and TaqMan probes for the IAP-family and glutaraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were synthesized at a commercial laboratory (PerkinElmer Cetus). The primers and TaqMan probes used were as follows. The sequence of the forward primer for survivin mRNA was 5′-TGCCTGGCAGCCCTTTC-3′ and that of the reverse primer was 5′-CCTCCAAGAAGGGCCAGTTC-3′; the TaqMan probe was 5′-CAAGGACCACCG CATCTCTACATTC-3′. For cIAP1 mRNA, the forward primer was 5′-CAGCCTGAGCAGCTTGCAA-3′ and the reverse primer was 5′-CAAGCCACCAT CACAACAAAA-3′; the TaqMan probe was 5′-TTT ATTATGTGGGTCGCAATGATGATGTCAAA-3′. For cIAP2 mRNA, the forward primer was 5′-TCCGTCAAGTTCAAGCCAGTT-3′ and the reverse primer was 5′-TCTCCTGGGCTGTCTGATGTG-3′; the TaqMan probe was 5′-CCCTCATCTACTTGAA CAGCTGCTAT-3′. The forward primer for NAIP mRNA was 5′-GCTTCACAGCGCATCGAA-3′ and the reverse primer was 5′-GCTGGGCGGATGCT TTC-3′; the TaqMan probe was 5′-CCATTTAAAC CACAGCAGAGGCTTTAT-3′. The forward primer for XIAP mRNA was 5′-AGTGGTAGTCCTGTTT CAGCATCA-3′ and the reverse primer was 5′-CCGCACGGTATCTCCTTCA-3′; the TaqMan probe was 5′-CACTGGCACGAGCAGGGTTTCTT TATACTG-3′. Finally, the forward primer for GAPDH mRNA was 5′-GAAGGTGAAGGTCG GAGT-3′ and the reverse primer was 5′-GAA GATGGTGATGGGATTTC-3′; the TaqMan probe was 5′-CAAGCTTCCCGTTCTCAGCC-3′. The conditions for one-step RT-PCR were as follows: 30 min at 48°C (stage 1, reverse transcription), 10 min at 95°C (stage 2, RT inactivation and AmpliTaq Gold activation), and then 40 cycles of amplification for 15 sec at 95°C and 1 min at 60°C (stage 3, PCR). The expression of survivin and other IAP-family proteins was quantitated according to a method described elsewhere [13]. Briefly, the intensity of the reaction was evaluated from the quantity of total RNA in Raji cells (ng) corresponding to the initial number of PCR cycles to reveal the linear increase in reaction intensity (threshold cycle) for each sample on a logarithmic standard curve. Data on the quantity of RNA (ng) for the IAP family was normalized using the data for GAPDH in each sample, and then the ratio to the mean value of control subjects was calculated and compared.

Immunohistochemistry for Survivin and Proliferating Cells

Tissue sections (4 μm thick) of bone marrow from the control, AML, ALL, and AMLL cases were cut on slides covered with adhesive. The sections were deparaffinized, and endogenous peroxidase was quenched with 1.5% hydrogen peroxide in methanol for 10 min. Antibodies were applied to identify survivin and to characterize proliferating cells. The primary antibodies included polyclonal rabbit antibody against human survivin (SURV 11-A, Alpha Diagnostic International, Inc., San Antonio, TX) and monoclonal antibody Ki-67 (DAKO). All sections were developed using biotin-conjugated secondary antibodies against rabbit IgG or mouse IgG followed by a sensitive peroxidase-conjugated streptavidin system (DAKO) with DAB as the chromogen. Negative control staining was performed using rabbit or mouse immunoglobulin of irrelevant specificity substituted for the primary antibody. The proportion of Ki-67-positive cells was determined in the same way as the proportion of TUNEL-positive cells.

Statistical Analysis

Statistically significant differences in the quantitative analysis were determined using the Mann–Whitney *U*-test for comparisons between the control, AML, ALL, and AMLL samples.

RESULTS

Clinicopathological Characteristics of Cases With Acute Mixed Lineage Leukemia

To determine the clinicopathological characteristics of cases with AMLL, the clinical data for cases including laboratory findings are summarized in Table I. As indicated by the flow-cytometric data, bone marrow blasts in these cases exhibited a high frequency of B-cell lineage antigen (CD19) and myeloid cell marker (CD13 and/or CD 33) expression. Thus, blasts of these cases were "biphenotypic." Chromosomal abnormalities were identified in 5 cases (cases 1, 3, 6, 7, and 8), and the Philadelphia chromosome was identified in two cases (cases 1 and 7). Although abnormalities involving chromosome 11q were identified in two cases (cases 1 and 8), the molecular rearrangement of the *mixed lineage leukemia* (*MLL*) gene located on chromosome 11q23 [32,33] was not observed at the chromosome level.

In spite of AML- and ALL-directed therapy (cytarabine, vincristine, etoposide, adriamycin, predonin, etc.), five patients failed to exhibit complete hematological remission, having blast persistence in bone marrow above 10%. Although complete remission could be induced by chemotherapy in four cases (cases 3, 6, 7, and 8), relapse with leukemic blast proliferation occurred within 6 months in two cases (cases 3 and 6, Table II). Overall, most cases exhibited a poor prognosis and the survival times after diagnosis were shorter than 14 months for 5 cases. However, one patient who received a bone marrow transplant (case 6) and the other patients who received chemotherapy (cases 7 and 8) lived.

Double Immunostaining for Myeloid and Lymphoid Cell Markers on AMLL Cells

To confirm the biphenotypic nature of blasts in the AMLL samples, double immunostaining for myeloid and lymphoid cell markers was performed. The majority of AMLL cells exhibited positive signals for B-cell markers such as CD20 or CD79a, while the myeloid cell marker (myeloperoxidase) was partially observed for many of the cases examined

TABLE II. Treatment and Outcome of Cases With Adult AMLL*

Case no.	First treatment	Response and status	Second treatment	Response status	Survival (months)
1	A-VVV	Failure	H-CPM/VP-16	Failure	3
2	H-CPM/VP-16, H-AraC+MIT	Failure	TBI + CPM	Failure	5
3	DCM, H-AraC+MIT	CR, relapse	A-VVV, H-AraC	Failure	6
4	L-AdVP, MVP	Failure	B-VVV, H-CPM/VP-16, H-AraC, L-AdVP	Failure	11
5	AdVP	Failure	A-VVV, VP-16, CAG	Failure	14
6	DC, A-VVV, H-CPM/VP-16	CR, relapse	H-AraC + MIT, BMT	CR and alive	>6
7	A-VVV	CR	H-AraC + MTX	CR and alive	>6
8	CAG	CR	DC	CR and alive	>9

*Abbreviations: A-VVV, AraC (cytarabine) + VCR (vincristine) + VLB (vinblastine) + VP-16 (etoposide); H-CPM, high-dose CPM (cyclophosphamide); H-AraC, high-dose AraC; MIT, mitoxantrone; TBI, total body irradiation; DCM, DNR (daunorubicin) + AraC + 6-MP (mercaptopurine); CR, complete remission; L-AdVP, L-Asp (L-asparaginase) + ADR (doxorubicin) + VCR + PDN (predonin) + CPM; MVP, MIT + VP-16 + PDN; B-VVV, BHAC (enocitabine) + VCR + VLB + VP-16; CAG, AraC + ACR (acurabicin) + G-CSF (lenograstim); DC, DNR + AraC; BMT, bone marrow transplantation; MTX, methotrexate.

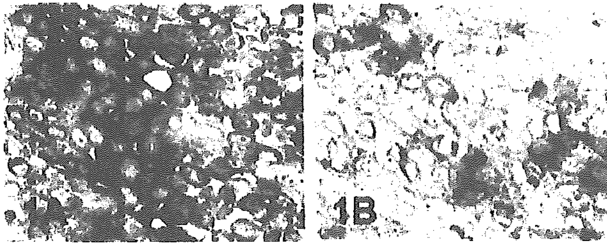

Fig. 1. Double immunostaining for a B-cell marker (CD79a) and myeloid cell marker (myeloperoxidase) in cases with AMLL (A, case 8; and B, case 6; original magnification 400×). Note that the majority of blasts stained positively for CD79a (brown) and a portion of them also stained positive for myeloperoxidase (blue) in both cases. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(Fig. 1A for case 8 and Fig. 1B for case 6). These findings were consistent with the flow-cytometric analytical data shown in Table I.

Apoptotic Frequency and Proliferation Activity of Acute Mixed Lineage Leukemia

To identify the apoptotic and proliferative cells present in the bone marrow samples, the TUNEL method and immunohistochemistry for Ki-67 were performed on paraffin-embedded sections. As expected from our previous studies [7,10], the frequency of apoptosis was significantly lower in AML (median, 0.769; range, 1.06–0.219) ($P < 0.001$) and ALL bone marrow cells (median, 0.543; range, 1.18–0.072) ($P < 0.01$) than control cells (median, 2.03; range, 2.81–0.848), and the proliferative cell ratio in AML/ALL bone marrow (median, 39.7; range, 47.8–32.4/median, 45.9; range, 71.9–34.2) was significantly higher than that in control cases (median, 19.2; range, 24.3–10.0) ($P < 0.0001$ and $P < 0.001$, respectively). As shown in Table III, AMLL cells exhibited a tendency similar to AML and ALL cells in that the apoptotic ratio (median, 0.176; range, 1.69–0.021) was significantly lower than the control ($P < 0.01$) and the proliferative cell ratio (median, 26.7; range, 49.1–18.3) was significantly higher ($P < 0.01$). However, AMLL cells exhibited a relatively lower apoptotic index and also significantly lower proliferative index compared with the AML ($P < 0.05$) or ALL samples ($P < 0.05$).

Expression of IAP-Family Proteins Determined by Real-Time Quantitative PCR

To quantitate the mRNA expression levels of the IAP-family members in AMLL cells, real-time quantitative RT-PCR was performed using bone marrow samples from control, AML, ALL, and AMLL cases.

TABLE III. Apoptotic Frequency and Proliferation Activity of Bone Marrow Cells From Control and Acute Leukemia Cases*

	TUNEL$^+$ cell ratio (%)	Ki-67$^+$ cell ratio (%)
Cases	Median (max-min)	Median (max-min)
Control	2.03 (2.81–0.848)^{a,b,c}	19.2 (24.3–10.0)^{d,e,f}
AML	0.769 (1.06–0.219)^{a}	39.7 (47.8–32.4)^{d,g}
ALL	0.543 (1.18–0.072)^{b}	45.9 (71.9–34.2)^{e,h}
AMLL	0.176 (1.69–0.021)^{c}	26.7 (49.1–18.3)^{f,g,h}

*Values indicate the median, maximum, and minimum. Differences were significant between the TUNEL-positive cell ratio for control and AML ($^aP < 0.001$), control and ALL ($^bP < 0.01$), and control and AMLL ($^cP < 0.01$) as seen by the Mann–Whitney *U*-test. The Ki-67-positive cell ratio exhibited significant differences between control and AML ($^dP < 0.0001$), control and ALL ($^eP < 0.001$), control and AMLL ($^fP < 0.01$), AML and AMLL ($^gP < 0.05$), and ALL and AMLL ($^hP < 0.05$) as seen by the Mann–Whitney *U*-test.

As shown in Fig. 2, the expression of survivin ($P < 0.05$), cIAP1 ($P < 0.05$), NAIP ($P < 0.01$), and XIAP ($P < 0.01$) exhibited significant up-regulation in AMLL compared with the controls. The mRNA for survivin ($P < 0.05$) showed significantly higher levels of expression in AMLL than AML, while the expression levels of survivin ($P < 0.05$), NAIP ($P < 0.05$), and XIAP ($P < 0.05$) in AMLL were significantly higher than those in ALL.

In summary, survivin expression in AMLL was significantly higher than the expression in control, AML, and ALL. The expression level of cIAP1 in AMLL was significantly higher than that in control, but similar with the expression in AML and ALL. Regarding cIAP2, the AMLL cases exhibited stronger expression than the control, AML, and ALL samples although the differences were not significant. NAIP expression in AMLL was significantly higher than control and ALL. The expression level of XIAP in AMLL was significantly higher than control and ALL but similar with AML. No remarkable differences were found between IAP protein expression and patients' age, sex, phenotype, or genotype for AMLL, although further analysis would be necessary because the number of cases was rather small.

These results indicate that the overall expression of IAP-family proteins in AMLL subjects tended to be higher than that for the control, AML, or ALL samples. Specifically, survivin expression in AMLL was significantly higher than that for the control, AML, and ALL samples.

Immunohistochemical Detection of Survivin in the Bone Marrow of AMLL Subjects

To investigate the distribution of survivin, immunohistochemical staining was performed on bone

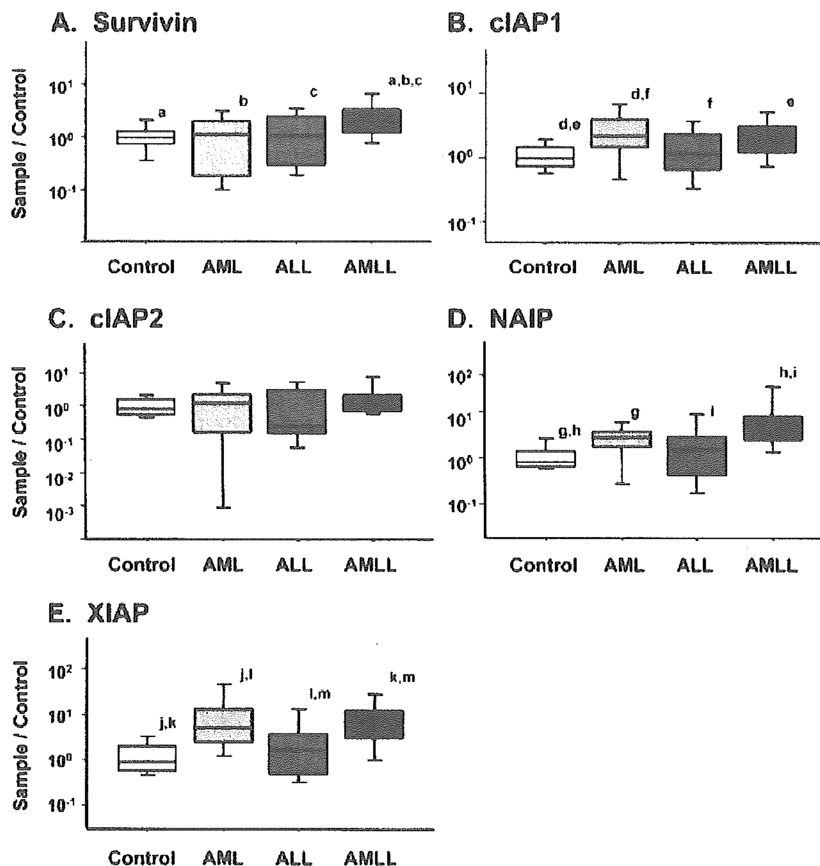

Fig. 2. Expression of IAP-family proteins in control bone marrow and acute leukemias determined by real-time quantitative RT-PCR. The relative intensity was calculated as (intensity of the reaction of IAP-family members [total Raji RNA, ng])/(intensity of the reaction of GAPDH [total Raji RNA, ng]). The intensities of the expressions from the AML, ALL, and AMLL samples are indicated as the ratios to the intensity of the control subjects. The box–bar graphs indicate the value of the control, AML, ALL, and AMLL cases: the bars indicate the 90th and 10th percentiles, and the box indicates the 75th to 25th percentiles. Differences were significant between samples as seen by the Mann–Whitney *U*-test as follows: (A) survivin—control and AMLL ($^{a}P < 0.01$), AML and AMLL ($^{b}P < 0.05$), and ALL and AMLL ($^{c}P < 0.05$). (B) cIAP1—control and AML ($^{d}P < 0.01$), control and AMLL ($^{e}P < 0.05$), and AML and ALL ($^{f}P < 0.05$). (C) cIAP2—differences were not significant. (D) NAIP—control and AML ($^{g}P < 0.05$), control and AMLL ($^{h}P < 0.01$), and ALL and AMLL ($^{i}P < 0.05$). (E) XIAP—control and AML ($^{j}P < 0.01$), control and AMLL ($^{k}P < 0.01$), AML and ALL ($^{l}P < 0.05$), and ALL and AMLL ($^{m}P < 0.05$).

marrow samples from AMLL subjects. As we previously showed [13], survivin was detected in only a few scattered myeloid cells in the control bone marrow samples and subcellular localization was mainly cytoplasmic but partly nuclear. The staining pattern and intensity in the control bone marrow was constant between different samples. All of the AMLL samples showed positive staining for survivin, although the staining intensity and frequency varied for each case. At the cellular level, survivin signals in AMLL cells were predominantly localized in the nucleus and also weakly in the cytoplasm (Fig. 3A). However, one case exhibited prominent cytoplasmic staining with mildly positive staining in the nucleus (Fig. 3B). The tissue sections that reacted with preimmune rabbit antibody of nonrelevant specificity showed no significant staining for any of the samples (not shown).

DISCUSSION

AMLL blasts are expected to possess more immature or intermediate characters of AML and ALL blasts because they express both myeloid and lymphoid phenotypes. Regarding the expression of survivin in myeloid neoplasms, previous studies have revealed the significant expression of survivin in AML [34,35]. Adida et al. [35] reported that survivin expression frequently occurs in AML, detecting it in 60% of a series of 125 patients analyzed, and survivin expression was found to be an unfavorable prognostic factor. In contrast, in lymphoid neoplasms, several

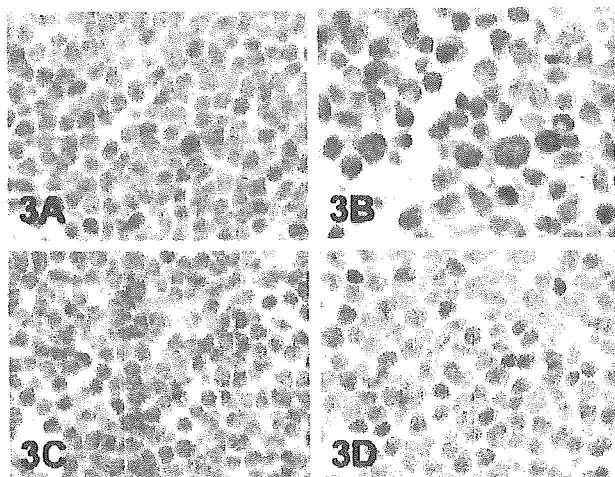

Fig. 3. Immunostaining for survivin in the bone marrow of AMLL (A, case 5; and B, case 8) in comparison with AML (C) and ALL (D) (original magnification 400×). Development was performed using the peroxidase–DAB system (brown) with hematoxylin counterstaining. Note the positive signals in the nucleus as well as the cytoplasm of AMLL cells (A) in contrast to the cytoplasmic staining (B). AML (C) and ALL (D) cases exhibited nuclear and partial cytoplasmic staining. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

studies investigated the dynamics of survivin expression in association with cell proliferation. The in vitro data on mononuclear cells from peripheral blood or bone marrow indicated that B-CLL cells expressed survivin in concert with CD40 and that survivin was the only IAP whose expression was induced by the CD40 ligand (CD40L) [36]. CD40 belongs to the TNF receptor superfamily [37], and its stimulation rescues B-CLL cells from apoptosis and induces proliferation [38]. We recently found that ALL as well as CLL cells exhibited significant expression of survivin and cIAP2 [13]. Thus, both in myeloid and lymphoid neoplasms, IAPs are expressed and seemed to influence the prognosis of patients. Therefore, we can imagine that IAPs would have functions also in AMLL blasts; however, little is known about the potential roles of survivin and other IAPs in the pathogenesis of AMLL.

A major problem with leukemia treatment is drug resistance to chemotherapeutic agents, which may already be present upon diagnosis or after chemotherapy for minimal residual blasts. Resistance originates from genetic or epigenetic mutations during growth of the leukemic clone. Anti-apoptosis mechanisms, alterations of tumor suppressor genes, altered immunogenicity, and drug-resistance mechanisms act in combination [39]. AMLL exhibits strong resistance against chemotherapy, resulting in poor patient prognosis [40,41]. In the present study, expression levels of IAPs in AMLL blasts were higher than those in control samples. Furthermore, several IAPs, such as survivin, NAIP, and XIAP, exhibited stronger expression in AMLL compared with conventional acute leukemias. Thus, the IAP expression level is one criterion that can be used to explain the strong drug resistance in this category of leukemia. The IAP might function probably via the inhibition of caspase-dependent apoptotic signaling. Although we have yet to clarify the caspase-independent pathway of apoptosis in AMLL, the findings of the present study suggest that the regulation of IAPs may become a possible target of AMLL therapy in the future.

In addition to its anti-apoptotic function, survivin also helps regulate cell-cycle progression during mitosis [20]. The highly proliferative activity of AMLL bone marrow cells as well as AML/ALL cells might be associated with survivin expression. As for the expression of IAPs in AML/ALL, the present study found strong expression in some cases and control levels in others, suggesting that AML/ALL cases are heterogeneous in terms of IAP expression.

The human *MLL* gene is involved in about 50 different chromosomal translocations associated with the acute leukemia phenotype [42]. Although chromosomal rearrangement involving chromosome 11q23 was not identified, the cases in the present study were not examined for the presence of *MLL* gene rearrangement by PCR analysis at the DNA level. Further studies are necessary to clarify the interaction of the *MLL* gene and IAP-family genes in association with apoptotic signaling in AMLL blasts.

In conclusion, we showed that strong expression of IAPs, especially survivin and NAIP, occurs in AMLL. Further studies are warranted to clarify the regulatory mechanisms of IAP expression in AMLL in association with drug resistance in this leukemia.

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Early Immune Reaction after Reduced-Intensity Cord-Blood Transplantation for Adult Patients

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Background. To investigate immune reactions after reduced-intensity cord-blood transplantation (RI-CBT).
Materials and Methods. We reviewed medical records of 57 adult RI-CBT recipients. Preparative regimen comprised fludarabine, total-body irradiation, and either melphalan (n=51) or busulfan (n=6). Graft-versus-host disease (GvHD) prophylaxis was cyclosporine. PostRI-CBT immune reactions were classified according to time course: preengraftment immune reactions (PIR), engraftment syndrome (ES), and GvHD.
Results. Forty-five patients achieved engraftment at a median of day 19. PIR was characterized by high-grade fever and weight gain and developed on a median of day 9 in 35 of the 45 evaluable patients, including 3 who did not achieve engraftment. PIR subsided spontaneously in 12 patients, whereas corticosteroids were required in the other 23. ES and grade I to IV acute GvHD developed in 36 and 29 patients, respectively. GvHD could not be distinguished from preceding PIR or ES in 10 patients. Causes of the 32 nonrelapse mortalities included GvHD (n=5) and PIR (n=1). There were no significant differences in relapse and nonrelapse deaths between patients with PIR and those without it (18% vs. 5%, and 60% vs. 65%, respectively).
Conclusions. Immune reactions after RI-CBT can be categorized into three distinct subtypes.

Keywords: Graft-versus-host disease, Engraftment syndrome, Preengraftment immune reaction, Allogeneic hematopoietic stem-cell transplantation, Nonmyeloablative stem-cell transplantation.

(*Transplantation* 2005;80: 34–40)

Cord-blood transplantation (CBT) is a promising approach for patients with advanced hematologic malignancies who lack a suitable donor. Cord blood has many theoretic advantages as a stem-cell source. Hematopoietic progenitors from cord blood are enriched in primitive stem cells, producing in vivo long-term repopulating stem cells (*1*). Another advantage of cord blood is immaturity of immune function. Long-lasting unresponsiveness and lack of proliferation of cord-blood lymphocytes on rechallenge with alloantigen might lead to reduced incidence of graft-versus-host disease (GvHD) after CBT (*2*), whereas a graft-versus-leukemia (GvL) effect might be maintained owing to the presence of precursor T and natural killer cells (*3, 4*). The feasibility of related and unrelated CBT has been demonstrated for pediatric patients (*5–8*), and the technique has been successfully applied to adults (*9–12*). Moreover, in adult patients with advanced hematologic malignancies, the feasibility of CBT using reduced-intensity preparative regimens (reduced-intensity CBT [RI-CBT]) has been demonstrated by us and other researchers (*13–16*).

Several types of immune reactions have been reported after allogeneic stem-cell transplantation (allo-SCT). With the exception of acute GvHD (*6, 17–19*), little information is available regarding immune reactions after CBT. The incidence and severity of acute GvHD after unrelated CBT have been low compared with those after allo-SCT from a matched unrelated donor or a mismatched family donor, despite the infusion of human leukocyte antigen (HLA)-mismatched graft (*20*); however, the reported incidence of grade II to IV acute GvHD varies from 25% to 72% (*5, 7–9, 11, 21–24*). Sanz et al. (*10*) reported that 21 of 22 adult CBT recipients developed grade I to IV acute GvHD and that median time to development of GvHD was 9 (range 4–14) days. Considering that median time to neutrophil engraftment was 25.5 (range 14–64) days, the majority of patients developed acute GvHD before engraftment. Similar findings have been reported by other groups (*14*). The circumstances of these immune reactions appear different from those seen in conventional allo-SCT.

Different immune reactions may occur after RI-CBT, and we postulated that characterization of the clinical features of these reactions in relation to engraftment would be useful. We investigated clinical features of immune reactions in 57 patients who underwent RI-CBT at Toranomon Hospital.

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PATIENTS AND METHODS

Patients

Fifty-seven patients underwent RI-CBT at Toranomon Hospital between January 2002 and August 2003. Patient characteristics and transplantation procedures are shown in Table 1. All patients had hematologic disorders or solid tumors that were incurable with conventional treatments and were considered inappropriate for conventional allo-SCT because of the lack of an HLA-identical sibling or a suitable unrelated donor, age greater than 50 years, or organ dysfunction. All patients provided written informed consent in accordance with the requirements of the institutional review board.

Transplantation Procedures

Cord-blood units that were matched for four or more of six HLA antigens and contained at least 2.0×10^7 nucleated cells/kg of recipient body weight before freezing were used. Cord-blood units were not depleted of T lymphocytes.

The preparative regimen comprised fludarabine 25 mg/m^2 on days −7 to −3, melphalan 80 mg/m^2 on day −2 (n=51) or busulfan 4 mg/kg for 2 days (n=6), and 4 or 8 Gy total-body irradiation (TBI) in 2 fractions on day −1 (Table 1). Granulocyte colony stimulating factor at 300 μg/m^2/day was administered intravenously from day 1 until neutrophil engraftment. Laboratory data including C-reactive protein (CRP) were obtained three times a week.

For GvHD prophylaxis, a continuous infusion of cyclosporine at 3 mg/kg from day −1 until toleration of oral administration was administered. Acute GvHD was graded according to the established criteria (*25*). Patients with grade II to IV acute GvHD were given 0.5 to 2.0 mg/kg per day of methylprednisolone. Treatment of immune reactions other than GvHD was at physicians' discretion. Management of infections was reported previously (*13*).

Chimerism Analysis

Chimerism was assessed using fluorescent in situ hybridization in sex-mismatched donor-recipient pairs. In sex-matched pairs, polymerase chain reaction for variable numbers of tandem repeats was used with donor cells detected at a sensitivity of 10% (*26*). Whole-blood and CD3+ cell chimerism was assessed at the time of granulocyte engraftment. When engraftment was delayed, chimerism was assessed on day 30. For those who died before engraftment, chimerism was assessed at least once during life.

Definition of Engraftment and Immune Reactions

Engraftment was defined as white blood cell count greater than 1.0×10^9/L or absolute neutrophil count greater than 0.5×10^9/L for 2 consecutive days. Graft failure was defined as peripheral cytopenia and marrow hypoplasia occurring later than day 60, accompanied by failure to detect donor markers using cytogenetic or molecular techniques.

The reported clinical presentation of engraftment or pre-engraftment syndrome (ES) varies, primarily because of the lack of uniform diagnostic criteria (*27*). When patients with no evidence of infection or adverse effects of medication exhibited skin eruption, diarrhea, jaundice (serum levels of

TABLE 1. Characteristics of patients receiving cord-blood transplantation

Characteristics	n=57
Age (yr)	
Median	56
Range	21–72
Sex	
Male	32
Female	25
Diagnosis	no. of patients
Cancer	
Acute lymphoblastic leukemia	
1st complete remission	1
Advanced disease	7
Acute myeloblastic leukemia	
1st complete remission	1
Advanced disease	20
Myelodysplastic syndrome	
Refractory anemia	1
Others	2
Chronic myeloid leukemia	
Advanced disease	1
Malignant lymphoma	
1st complete remission	1
Advanced disease	16
Multiple myeloma	
Advanced disease	1
Solid tumor	2
Bone marrow failure syndrome	
Severe aplastic anemia	4
Conditioning regimen	
Fludarabine/Busulfan/Total body irradiation 4 Gy	5
Fludarabine/Busulfan/Total body irradiation 8 Gy	1
Fludarabine/Melphalan/Total body irradiation 4 Gy	48
Fludarabine/Melphalan/Total body irradiation 8 Gy	3
Graft-versus-host disease prophylaxis	
Cyclosporin	57
Infused CD34+ cells ($\times10^5$/kg)	
Dose	2.9
Range	2.1–4.4
Body weight (kg)	
Median	53.8
Range	37.3–77.4
No. of HLA-A, B, and DRB1 mismatches	
0	1
1	8
2	48

HLA, human leukocyte antigen.

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total bilirubin>2.0 mg/dL), or body weight gain greater than 10% of baseline, these parameters were defined as immune reactions. Reactions were classified into the following three subtypes according to timing: pre-engraftment, peri-engraftment, and postengraftment. Immune reactions which developed 6 or more days before engraftment were defined as pre-engraftment immune reactions (PIR). Those within 5 days of engraftment were defined as ES. Others were defined as postES, which generally corresponded to acute GvHD. Acute and chronic GvHD were graded according to the consensus criteria (*25*, *28*). In the treatment of PIR, ES, and GvHD, response to corticosteroid was evaluated as reported previously (*29*).

Primary Endpoints and Statistical Analysis

The primary endpoint of this study was to investigate clinical characteristics of immune reactions after RI-UCBT. Immune reactions were divided into three categories: PIR, ES, and acute GvHD. The following variables were assessed: fever, serum levels of CRP, skin eruption, diarrhea, jaundice, central nervous system complications, weight gain greater than 10% of baseline, documented infections, and response to corticosteroid. The secondary endpoint was to investigate whether these reactions had a prognostic impact.

Overall survival (OS) and relapse-free survival (RFS) were determined using the Kaplan-Meier method. Final follow-up was conducted in July 2004, with a median follow-up of surviving patients being 16.0 (range 13.8–32.4) months. Surviving patients were censored on the last day of follow-up. ES and GvHD were analyzed in patients who achieved initial engraftment. Cumulative incidence of PIR, ES, GvHD, relapse, and nonrelapse-related mortality (NRM) were calculated using Gray's method (*30*), treating death without each type of immune reaction as a competing risk. A multivariate Cox proportional hazards model was used to identify independent and significant prognostic factors for OS and RFS. The variables entered in each analysis were patient age, sex, primary diseases, risks, number of transfused mononuclear cells, HLA-disparity, and dose of TBI. PIR and acute GvHD were included as a time-dependent covariate. A significance level of 5% was set as the limit for inclusion in the model. Prognostic factors that were significant at $P<0.05$ in the stepwise proportional model analysis were considered to be important in influencing survival.

RESULTS

Engraftment and Chimerism Analysis

Forty-five patients achieved engraftment. Median day of engraftment was day 19 (range 11–55). Cumulative incidences of engraftment and death without engraftment at day 100 were 79% and 18%, respectively (Fig. 1). Rescue of primary graft failure occurred in two patients after second RI-UCBT. The remaining 10 patients died before engraftment after a median of 24.5 (range 15–45) days. Causes of death included regimen-related toxicity (n=2), infection (n=6), progression of underlying disease (n=1), and multiple organ failure caused by pre-ES (n=1).

Chimerism data were obtained from 52 patients. Cumulative incidence of complete donor chimerism at day 60 was 97%, and median time to complete donor chimerism was 22 (range 8–54) days. Complete donor chimerism was documented in the seven patients who died of NRM before engraftment.

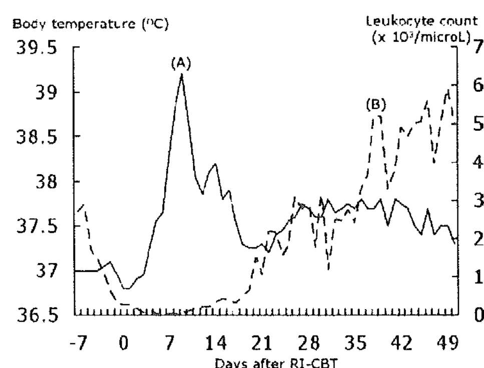

FIGURE 1. Typical clinical course of pre-engraftment immune reaction (PIR). High-grade fever developed on a median of day 9, during severe neutropenia. (A) Body temperature; (B) leukocyte count. RI-CBT, reduced-intensity cord blood transplantation.

Pre-engraftment Immune Reaction

Twelve patients who developed documented infection before engraftment were excluded from the analysis of PIR. Thirty-five of the remaining 45 (78%) patients developed PIR on a median of day 9 (range 6–13). Typical clinical courses of PIR are shown in Figure 2. PIR was observed in three patients who had never engrafted as well as those who had achieved engraftment.

Compared with ES and GvHD, body weight gain, high-grade fever, and elevation of serum levels of CRP were more frequent in PIR. In contrast, jaundice was more common in ES and GvHD than in PIR (Table 2). Histologic examination of the skin was conducted in six patients. Infiltration of mononuclear cells was not prominent in any of the six patients. Common findings were vascular dilatation (n=4) and intercellular edema in the dermis (n=4).

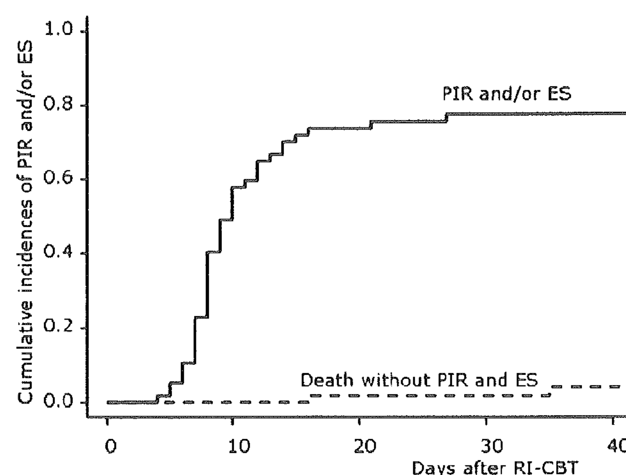

FIGURE 2. Days to PIR or engraftment syndrome (ES), treating death without these complications as a competing risk. Cumulative incidence of PIR or ES was 78%.

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