Table 4. Effect of donor HTLV-I serostatus on transplantation outcomes

		Univariable analysis		Multivariable analysis	
Outcome	**Number***	**Hazard ratio (95% CI)**	***P***	**Hazard ratio (95% CI)**	***P***
Overall survival†					
Donor HTLV-I antibody positive	43/68	1.00	Reference	1.00	Reference
Donor HTLV-I antibody negative	52/88	0.90 (0.60-1.35)	.603	0.83 (0.54-1.28)	.395
Treatment-related mortality‡					
Donor HTLV-I antibody positive	20/64	1.00	Reference		
Donor HTLV-I antibody negative	37/86	1.51 (0.88-2.58)	.133		
Disease-associated mortality§					
Donor HTLV-I antibody positive	19/64	1.00	Reference	1.00	Reference
Donor HTLV-I antibody negative	13/86	0.44 (0.22-0.89)	.022	0.43 (0.21-0.90)	.026

CI indicates confidence interval; and HTLV, human T-cell leukemia virus.

*Number of events/number of evaluable patients.

†Other variables considered in the multivariable analysis were disease status before transplantation, type of GVHD prophylaxis, and type of graft source. Variables significantly associated with overall survival were disease status before transplantation and type of GVHD prophylaxis: not in complete remission versus complete remission (hazard ratio, 1.95; 95% CI, 1.17-3.24, $P = .010$); tacrolimus- versus cyclosporine-based (hazard ratio, 4.22; 95% CI, 1.58-11.26, $P = .004$).

‡Multivariable analysis was not performed because no variable was significantly associated with treatment-related mortality by univariable analysis.

§Other variables considered in the multivariable analysis were disease status before transplantation, type of GVHD prophylaxis, and type of graft source. The only variable significantly associated with disease-associated mortality was disease status before transplantation: not in complete remission versus complete remission (hazard ratio, 2.88; 95% CI, 1.01-8.24, $P = .049$).

transplantation outcomes by graft source was not feasible because the selection of graft source is an individual process strongly influenced by donor availability and disease status of patients. It should also be noted that the study period encompassed the developmental phase of cord blood transplantation in adults. Because rates of disease-associated death were similar irrespective of type of graft source, new strategies to reduce early treatment-related mortality would improve the results of alternative donor transplantations for ATL.

Another concern related to selection of graft source involves the use of HTLV-I-seropositive-related donors. Sibling donors for patients with ATL are frequently infected with HTLV-I, because mother-to-child transmission by breastfeeding is a major route of HTLV-I acquisition.[5,6] The use of HTLV-I–seropositive donors raises the risk of ATL development in donor-derived HTLV-I–infected cells under immunosuppressive conditions after transplantation,[41] whereas it may enhance the therapeutic effect by the adoptive transfer of viral-specific immunocompetent cells.[21] However, the latter possibility seems less likely because transplantation from HTLV-I–seropositive donors was associated with higher risk for disease-associated mortality in our study cohort. Given that donor-derived HTLV-I–specific cytotoxic T-cell response can be observed in transplantation from an HTLV-I–seronegative donor,[21] it is important to note that the magnitude of specific T-cell responsiveness to HTLV-I might widely differ among healthy HTLV-I carriers. The impairment of HTLV-1–specific T-cell responses was observed not only in patients with advanced ATL but also in a subpopulation of asymptomatic carriers, which was associated with insufficient control of HTLV-I.[42] Although whether donor anti–HTLV-I immunity can harness graft-versus-ATL responses is still elusive, further investigations are clearly needed to resolve this issue.

This study had inherent limitations that are common among observational studies: eligibility for transplantation, as well as choice of transplantation protocol, including the selection of graft source, was determined by the treating physicians of each institution; the confounding effect of some variables, such as disease subtype, could not be fully evaluated because of missing data, although adjustment for other key risk factors enabled as controlled a comparison as possible.

In conclusion, allogeneic HSCT is an effective treatment that confers long-term survival in selected patients with ATL, but at the cost of substantial risk of treatment-related mortality. Posttransplantation outcomes are influenced by recipient age, recipient sex, and disease status at transplantation, as well as by type of graft source. More definitive conclusions on the role of allografting in the therapeutic algorithm for ATL will be drawn from future prospective studies that aim to compare the survival outcomes after transplantation with those after conventional chemotherapy.

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Authorship

Contribution: M. Hishizawa, J.K., T.I., and T.U. reviewed and analyzed data and wrote the paper; J.K., K.M., and T.I. performed statistical analysis; A.U., S.T., T.E., Y. Moriuchi, R.T., F.K., Y. Miyazaki, M.M., K.N., M. Hara, M.T., S. Kai, and J.O. interpreted data and reviewed and approved the final manuscript; Y.A., R.S., and H.S. collected data from the JSHCT; T.K. and Y. Morishima collected data from the JMDP; T.N.-I. and S. Kato collected data from the JCBBN; and T.I. and T.U. designed the research and organized the project.

T.U., the senior author, died after acceptance of the final manuscript.

In addition to authors, other members who contributed data on allogeneic hematopoietic stem cell transplantation for adult T-cell

leukemia to the JSHCT, JMDP, and JCBBN are listed in the supplemental Appendix.

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ORIGINAL ARTICLE

Pretransplant serum ferritin and C-reactive protein as predictive factors for early bacterial infection after allogeneic hematopoietic cell transplantation

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Although fluoroquinolones or other antibiotics are commonly used to prevent bacterial infections after hematopoietic cell transplantation (HCT), because of the growing presence of multidrug-resistant microorganisms, it is important to identify patients who are more likely to benefit from antibacterial prophylaxis. To evaluate risk factors for early bacterial infection after allogeneic HCT, we retrospectively analyzed clinical data for 112 consecutive adult patients with hematological malignancies who received transplants without any antibacterial prophylaxis. The cumulative incidence of bacterial infection at 30 days after transplantation was 16%. Among various pre-transplant factors, only high serum ferritin (>700 ng/mL, 47 patients) and high C-reactive protein (CRP) (>0.3 mg/dL, 28 patients) levels were significantly associated with the development of bacterial infection in a multivariate analysis (hazard ratio (95% confidence interval): ferritin, 4.00 (1.32–12.17); CRP, 3.64 (1.44–9.20)). In addition, septic shock and sepsis with organ failure were exclusively observed in patients who had high ferritin and/or high CRP levels. These results suggest that pretransplant serum ferritin and CRP levels can be useful markers for predicting the risk of early bacterial infection after allogeneic HCT. It may be prudent to limit antibacterial prophylaxis to patients with predefined risk factors to ensure the safety of HCT with the use of fewer antibiotics.

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Introduction

Bacterial infection is an important cause of mortality and morbidity after autologous or allogeneic hematopoietic cell transplantation (HCT).[1,2] When neutropenic patients who receive cytotoxic chemotherapy are compared with HCT recipients, the risk of severe bacterial infection appears to be considerably higher in the latter because high-dose chemotherapy and/or TBI may cause severe mucosal damage that facilitates massive bacterial translocation under profound post-transplant immunosuppression. Therefore, the use of oral fluoroquinolones (FQs) or other antibiotics as antibacterial prophylaxis is strongly considered for HCT recipients, although significant variations have been reported among transplant centers and countries.[3–5]

Recently, the widespread emergence of FQ-resistant or multidrug-resistant microorganisms in hematology–oncology units has been suggested to compromise the effectiveness of routine antibacterial prophylaxis with FQs in patients undergoing cytotoxic chemotherapy or HCT.[6–14] In our center, the isolation rate of FQ-resistant Gram-negative bacilli was high (57.1%) during a period when FQs were routinely administered as antibacterial prophylactic agents; in particular, among isolated *Enterobacteriaceae* strains, 66.7, 33.3 and 22.2% were resistant to levofloxacin, piperacillin and ceftazidime, respectively.[8] In an attempt to reduce the emergence of antibiotic-resistant microorganisms, we stopped using any antibacterial prophylaxis in both autologous and allogeneic HCT recipients in 2004,[8] and found that this discontinuation of FQ prophylaxis, even in the setting of myeloablative allogeneic HCT did not significantly affect early mortality after transplantation.[15]

Another approach to balance the safety of HCT with judicious antibiotic use would be to limit the use of antibacterial prophylaxis to HCT recipients who are at high risk of bacterial infection, because a delay in antibiotic treatment may lead to serious complications after infectious episodes in such patients if prophylactic antibiotics are not administered. To identify the pretransplant characteristics of patients who are more likely to be susceptible to bacterial infection after allogeneic HCT, we conducted a single-center retrospective study with the clinical data

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of 112 consecutive allogeneic transplants for hematologic malignancies that were performed without antibacterial prophylaxis. As a potential biomarker for predicting bacterial infection, high levels of pretransplant ferritin levels have recently been shown to be associated with an increased incidence of bloodstream infection, as well as decreased overall survival and increased early mortality.[16] In addition, elevated pretransplant serum C-reactive protein (CRP) levels have been shown to be associated with an increased incidence of bacterial infection in the setting of chemotherapy for acute myeloid leukemia[17] and allogeneic transplantation.[18] Sato *et al.*[17] reported that pretreatment serum CRP levels of greater than 0.26 mg/dL were useful for predicting the incidence of documented infection in patients who received their first consolidation chemotherapy for acute myeloid leukemia. As these biomarkers are easy to measure and may be useful in clinical practice, in this study we explored the association between bacterial infection and these biomarkers as well as various patient characteristics.

Subjects and methods

Study population

The medical records of 137 consecutive adult patients with hematological malignancies who underwent T-cell replete allogeneic HCT from September 2004 to March 2009 at Kyoto University Hospital were reviewed. Patients who had active infections before the transplantation procedure ($n=23$) and those who had a recent history of autologous or allogeneic HCT within 1 year ($n=2$) were excluded; thus, a total of 112 patients were included in the analysis, without any duplication of subjects. Patients were considered to have standard-risk disease if they received a transplant without prior chemotherapy or in CR, whereas those who received a transplant in any other status were considered to have high-risk disease. This study was approved by the Ethics Committee of Kyoto University Graduate School and the Faculty of Medicine. Written informed consent for the transplantation protocol was obtained from all of the patients.

Prophylaxis monitoring and diagnosis of infection

A central venous catheter was inserted in the subclavian vein before the beginning of the conditioning regimens for all patients. Each patient was isolated in a single room equipped with a HEPA system from a day before the transplantation until at least 4 weeks after transplantation. Each patient was then maintained on a low-microbial diet and asked to take strict control measures under the supervision of the ward staff to prevent the acquisition of nosocomial pathogens. No bacterial prophylaxis was prescribed for these patients according to our institutional protocols.[8] Intravenous antibiotics with anti-pseudomonal activity were promptly administered in response to episodes of febrile neutropenia or suspected bacterial infections. Trimethoprim-sulfamethoxazole (trimethoprim; 160 mg/day, three times a week) was administered as prophylactic therapy for *Pneumocystis jiroveci* pneumonia from the day of admission until the day of transplantation, and this prophylaxis was reinitiated after the day of neutrophil engraftment. All patients received 200 or 400 mg of fluconazole and 400–1000 mg of acyclovir per day as prophylactic agents from the conditioning period until 30 days after transplantation. Prophylactic fluconazole and acyclovir were usually continued when patients were receiving steroid therapy for acute or chronic GVHD. For each febrile episode, 1 or 2 sets of blood samples were cultured, and cultures of specimens other than blood specimens and imaging examinations to search for the focus of infection were performed according to the clinician's judgment. Microbiologically documented infections included the presence of bloodstream bacterial infection or any other bacterial infection. Bloodstream bacterial infection was diagnosed when at least 1 of the following criteria was met: (1) the culture of blood obtained during a febrile episode tested positive at least once for bacterial organisms other than common skin contaminants, (2) the culture of blood obtained during a febrile episode tested positive for the same common skin contaminant in independent analysis conducted within an interval of 72 h, and (3) the blood culture tested positive at least once for a common skin contaminant, and the patient was diagnosed with septicemia with hypotension (systolic blood pressure, <90 mm Hg) and disseminated intravascular coagulation. Infections other than bloodstream infection were diagnosed when the following criteria were met: (1) sputum, urine or stool samples were found to contain pathogenic bacteria on at least two occasions, and (2) the patient showed symptoms of infection corresponding to those specimens. Septic shock and sepsis with organ failure were defined as severe infection.

Measurement of serum biomarkers (serum ferritin and C-reactive proteins)

Both serum ferritin and CRP levels were measured using peripheral blood samples obtained just before the start of the conditioning regimen. The serum ferritin concentration was measured by an immunoenzymometric assay (AIA-PACK FER, Tosoh Corporation, Tokyo, Japan) (normal range; $\leqslant 150$ ng/mL), and the serum CRP concentration was measured by a latex agglutination assay (N-Assay LA CRP-S, Nittobo, Tokyo, Japan) (normal range; $\leqslant 0.2$ mg/dL), according to the respective manufacturer's instructions.

Statistical analysis

The primary endpoint was the cumulative incidence of documented bacterial infections during the first 30 days after transplantation. Overall survival and treatment-related mortality were also analyzed as secondary endpoints. To eliminate the effect of a competing risk, the cumulative incidence was assessed using methods described elsewhere.[19] In the analysis of the cumulative incidence of bacterial infections and treatment-related mortality, a competing event was defined as death without an event of interest. The overall survival was estimated using the Kaplan–Meier method. We applied Fine and Gray's proportional hazards model for the sub-distribution of a competing risk to analyze the cumulative incidence of

bacterial infection and treatment-related mortality, and the Cox proportional hazards model for that of overall survival.[20] Factors with *P*-values of less than 0.10 in the univariate analysis were included in the multivariate analysis. Factors evaluated in the analysis included the recipient's age (≤50 or >50), recipient's sex (female or male), diagnosis (myeloid or lymphoid malignancies), disease status at transplant (standard risk or high risk), duration from diagnosis to transplant (≤1 or >1 year), duration from the last pretransplant cytotoxic chemotherapy to conditioning of transplant (no history of prior chemotherapy or >2, or ≤2 months), number of courses of prior cytotoxic chemotherapy (≤5 or >5), source of stem cells (related BM or peripheral blood, unrelated BM, or unrelated cord blood), conditioning regimen (conventional or reduced-intensity regimen), use of granulocyte CSF (G-CSF) (yes or no), serum ferritin levels (≤700 ng/mL, >700 ng/mL, or unknown), and serum CRP levels (≤0.3 or >0.3 mg/dL). We assessed the interaction between ferritin and CRP levels, using interaction terms between a ferritin category with scores of 0 (ferritin ≤700 ng/mL) and 1 (ferritin >700 ng/mL) and a CRP category with scores of 0 (CRP ≤0.3 mg/dL) and 1 (CRP >0.3 mg/dL). The cutoff point for the ferritin levels was the median value and that for the CRP levels was the higher tertile value. The correlation between ferritin and CRP levels was also tested by Pearson's correlation coefficient. *P*-values of less than 0.05 were considered statistically significant. All analyses were conducted using Stata software version 11 (StataCorp., College Station, TX, USA).

Results

Patient characteristics

The patient characteristics are shown in Table 1. The median age of the patients was 47 years (range, 18–66 years). The primary diseases in these patients were as follows: acute myeloid leukemia in 46 patients, acute lymphoblastic leukemia in 11, myelodysplastic syndrome in 16, chronic myelogenous leukemia in four, non-Hodgkin's lymphoma in 19, adult T-cell leukemia/lymphoma in 10, myeloproliferative disorder in four, and plasma-cell myeloma in two. Sixty-six patients (58.9%) had standard-risk disease. The source of stem cells used for HCT was related BM or peripheral blood in 40 patients (35.7%), unrelated BM in 52 (46.4%), and unrelated cord blood in 20 (17.9%). A conventional myeloablative regimen was used in 54 patients (48.2%), and G-CSF was used after HCT in 57 patients (50.9%). The number of patients with pretransplant serum ferritin levels of ≤700, >700 ng/mL, and unavailable were 49, 47 and 16, respectively, and the number of those with pretransplant serum CRP levels of ≤0.3 and >0.3 mg/dL were 84 and 28, respectively.

Table 1 Patient characteristics

Category	*Patients (n = 112)*
Age, years	
Median (range)	47 (18–66)
Sex, n (%)	
Male	49 (43.8)
Female	63 (56.3)
Diagnosis, n (%)	
Acute myeloid leukemia	46 (41.1)
Acute lymphoblastic leukemia	11 (9.8)
Myelodysplastic syndrome	16 (14.3)
Chronic myelogenous leukemia	4 (3.6)
Non-Hodgkin's lymphoma	19 (17.0)
Adult T-cell leukemia/lymphoma	10 (8.9)
Myeloproliferative disorder	4 (3.6)
Plasma-cell myeloma	2 (1.8)
Disease status at transplant, n (%)	
Standard risk	66 (58.9)
High risk	46 (41.1)
Source of stem cells, n (%)	
Related bone marrow or peripheral blood	40 (35.7)
Unrelated bone marrow	52 (46.4)
Unrelated cord blood	20 (17.9)
Conditioning regimen, n (%)	
Conventional-intensity regimen	54 (48.2)
BU/CY	10
TBI/CY-based regimen	44
Reduced-intensity regimen	58 (51.8)
Flu/BU ± TBI	23
Flu/Mel ± TBI	34
Flu/TT	1
Use of G-CSF, n (%)	
Yes	57 (50.9)
No	55 (49.1)
Duration from diagnosis to transplant, n (%)	
≤1 year	57 (50.9)
>1 year	55 (49.1)
Duration from the last pretransplant cytotoxic chemotherapy to conditioning of transplant, n (%)	
No history of previous cytotoxic chemotherapy or >2 months	68 (60.7)
≤2 months	44 (39.3)
Number of courses of previous cytotoxic chemotherapy, n (%)	
≤5 courses	60 (53.6)
>5 courses	52 (46.4)
Pretransplant serum ferritin level (ng/mL)	
Median (range)	694.6 (34.7–12079.1)
Pretransplant serum CRP level (mg/dL)	
Median (range)	0.1 (0.0–4.6)

Abbreviations: Flu = fludarabine; Mel = melphalan; TT = thiotepa, G-CSF = granulocyte CSF.

Documented bacterial infections

A total of 19 episodes of bacterial infections were documented during the first 30 days after HCT; these included 18 episodes of bloodstream infections and 1 of pneumonia. No patient had more than 1 episode of bacterial infection within 30 days after HCT. The bacterial organisms associated with the documented infections are listed in Table 2. The detected bacterial organisms were mainly Gram-negative bacilli (*n* = 16, 84.2%), 15 of which (93.6%) were sensitive to FQs.

Table 2 Documented bacterial organisms within 30 days after transplantation

Category	*Bacterial isolates*
Gram-positive cocci (*n*)	*Enterococcus faecium* (2) *Streptococcus epidermidis* (1)
Gram-negative bacilli (*n*)	*Klebsiella pneumoniae* (5) *Escherichia coli* (4) *Pseudomonas aeruginosa* (2) *Klebsiella oxytoca* (1) *Enterobacter cloacae* (1) *Capnocytophaga species* (1) *Prevotella intermedia* (1) *Bacteroides thetaiotaomicron* (1)

P. aeruginosa was detected in the sputum of one patient with pneumonia. Other organisms were detected in blood culture bottles.

The cumulative incidence of bacterial infections was 16% (95% confidence interval (CI), 10–24%). Among confounding factors that were potentially associated with bacterial infection, only high pretransplant serum ferritin (>700 vs ⩽700 ng/mL) and high CRP (>0.3 vs ⩽0.3 mg/dL) levels were significantly associated with the development of bacterial infection in the multivariate analysis (hazard ratio (95% CI): ferritin, 3.97 (1.35–11.69), $P=0.012$; CRP, 3.63 (1.45–9.10), $P=0.006$) (Table 3). Even when serum ferritin and CRP levels were treated as continuous variables, their impact remained significant. Although there was no correlation between ferritin and CRP levels ($P=0.062$), we analyzed the impact of high ferritin levels in subgroups of patients with either high (>0.3 mg/dL) or low CRP levels (⩽0.3 mg/dL), to exclude the effect of inflammation on ferritin levels. We obtained almost consistent results in both groups (hazard ratio (95% CI): CRP >0.3 mg/dL, 3.67 (0.87–15.63), $P=0.078$; CRP ⩽0.3 mg/dL, 4.12 (0.86–19.64), $P=0.076$). Furthermore, no interaction was observed between the ferritin and CRP categories ($P=0.949$). Next, we re-evaluated the risk of bacterial infection with the combination of these two risk factors (ferritin and CRP levels). Figure 1 shows the cumulative incidence of bacterial infection for patients divided into three risk groups according to this model. The cumulative incidences of bacterial infections were 5.3% (95% CI; 1.0–15.7%) in patients without any risk factors ($n=39$), 20.5% (95% CI; 10.1–33.3%) in those with 1 factor ($n=44$), and 53.8% (95% CI; 24.8–76.0%) in those with two factors ($n=13$). The hazard ratios for 1 and 2 risk factors relative to no risk factors in the multivariate analysis were 4.04 (95% CI, 0.88–18.62) and 14.68 (3.02–71.30), respectively. Among patients with bacterial infections, septic shock or organ failure was observed in one patient with two risk factors and four patients with one risk factor, but not in any patients with no risk factors.

Overall survival and treatment-related mortality

Next, we evaluated the impact of the ferritin and CRP levels on other endpoints in 96 patients for whom data on ferritin levels were available (Figures 2 and 3). The median duration of follow-up was 23 months (range, 2.2–54.9). With regard to overall survival, only a high ferritin level (hazard ratio (95% CI): 2.47 (1.19–5.11), $P=0.015$) and a duration of less than 2 months from the last cytotoxic chemotherapy to the conditioning for transplant (hazard ratio (95% CI): 2.16 (1.10–4.26), $P=0.026$) were significant variables in the multivariate analysis. The causes of death are shown in Table 4. Interestingly, seven patients among those with high ferritin levels died within 100 days (causes of death: acute GVHD, $n=2$; infection, $n=3$; hepatic veno-occlusive disease, $n=1$; organ failure, $n=1$), whereas none of the patients with low ferritin levels died. With regard to treatment-related mortality, only ferritin and CRP levels were adversely associated with higher treatment-related mortality in the multivariate analysis (hazard ratio (95% CI): ferritin, 5.21 (1.41–19.30), $P=0.013$; CRP, 5.76 (1.70–19.48), $P=0.005$).

Discussion

In our cohort of 112 patients with hematologic malignancies who underwent allogeneic HCT without antibacterial prophylaxis, we found that only high serum ferritin and high CRP levels before transplantation were significant risk factors for the post-transplant development of bacterial infection; patients with high ferritin levels and those with high CRP levels had an almost 4-fold higher risk of bacterial infection than those with low ferritin levels or those with low CRP levels. In addition, although severe complications associated with bacterial infection were observed in five patients with high ferritin levels and/or high CRP levels, none were seen in patients with low ferritin and low CRP levels. These results suggest that pretransplant serum ferritin and CRP levels may be useful markers for predicting the risk of early bacterial complications after allogeneic HCT.

An association between iron overload and bacterial or fungal infection has been shown in hereditary and secondary hemochromatosis.[21,22] With regard to HCT, Pullarkat *et al.*[16] reported that ferritin levels of ⩾1000 ng/mL were associated with a 2-fold higher risk of bloodstream infection compared with patients with ferritin levels of <1000 ng/mL in myeloablative HCT. In agreement with their finding, in this study, ferritin levels of >700 ng/mL were associated with a 4-fold increased risk compared with the risk in patients with levels of ⩽700 ng/mL. An increase in plasma non-transferrin-bound iron (NTBI) is considered to have an important role in the adverse effect of iron overload on bacterial infection. Under normal conditions, toxic reactions due to the production of NTBI are prevented by circulating transferrin, which forms a compound with Fe^{3+}.[23] However, plasma NTBI increases to a measurable level in patients with iron overload because transferrin is almost saturated with Fe^{3+}.[24] The inhibition of iron utilization in erythrocytes by chemotherapeutic agents and irradiation further increases NTBI levels.[25] Hydroxyl radical reactions by NTBI exacerbate mucosal damage caused by chemotherapeutic agents and irradiation, which allows bacterial organisms to enter through circulation.[26] In addition, iron is an important nutrient for the proliferation of bacteria and fungi.[27] In the HCT

Table 3 Univariate and multivariate analyses of factors that are potentially associated with documented bacterial infection

Category	*Number*	*Univariate analysis*		*Multivariate analysis*	
		Hazard ratio (95% CI)	P-*value*	*Hazard ratio (95% CI)*	P-*value*
Age, years					
⩽50	10/64	1.00	Reference		
>50	9/48	1.27 (0.51–3.17)	0.608		
Sex					
Female	9/49	1.00	Reference		
Male	10/63	0.80 (0.32–2.00)	0.632		
Diagnosis					
Myeloid malignancies	13/72	1.00	Reference		
Lymphoid malignancies	6/40	0.86 (0.33–2.26)	0.766		
Disease status at transplant					
Standard risk	11/66	1.00	Reference		
High risk	8/46	1.13 (0.45–2.84)	0.795		
Source of stem cells					
Related bone marrow or peripheral blood	6/40	1.00	Reference		
Unrelated bone marrow	8/52	0.88 (0.30–2.59)	0.816		
Unrelated cord blood	5/20	1.84 (0.56–6.05)	0.318		
Conditioning regimen					
Conventional-intensity regimen	8/54	1.00	Reference		
Reduced-intensity regimen	11/58	1.48 (0.57–3.79)	0.419		
Use of G-CSF					
No	10/55	1.00	Reference		
Yes	9/57	0.98 (0.39–2.45)	0.966		
Duration from diagnosis to transplant					
⩽1 year	9/57	1.00	Reference		
>1 year	10/55	1.33 (0.53–3.34)	0.550		
Duration from the last pretransplant cytotoxic chemotherapy to conditioning of transplant					
No history of previous cytotoxic chemotherapy or >2 months	13/68	1.00	Reference		
⩽2 months	6/44	0.76 (0.29–2.03)	0.589		
Number of courses of previous cytotoxic chemotherapy					
⩽5 courses	9/60	1.00	Reference		
>5 courses	10/52	1.44 (0.57–3.64)	0.441		
Serum ferritin level					
⩽700 ng/mL	5/49	1.00	Reference	1.00	Reference
>700 ng/mL	14/47	4.04 (1.35–12.05)	0.012	3.97 (1.35–11.69)	0.012
Not available	0/16	—	—	—	—
Serum CRP level					
⩽0.3 mg/dL	10/84	1.00	Reference	1.00	Reference
>0.3 mg/dL	9/28	3.38 (1.36–8.39)	0.009	3.63 (1.45–9.10)	0.006

Abbreviations: CI = confidence intervals; G-CSF = granulocyte CSF.

setting, the ability of NTBI to induce the proliferation of *Staphylococcus epidermidis* has been shown in an *in vitro* study using the serum of patients undergoing HCT.[28]

In addition to the adverse impact of iron overload on early infection-related complications, several studies have suggested that high ferritin levels are adversely associated with overall survival and treatment-related mortality.[16,29–31] In agreement with these studies, our results showed that high ferritin levels are associated with a 2.5-fold increased risk of overall mortality and a 5-fold increased risk of higher treatment-related mortality, compared with low ferritin levels. These studies collectively suggest that iron overload is an important and strong prognostic factor in various clinical outcomes of allogeneic HCT.

Recently, an association between iron chelation therapy and longer overall survival was shown in patients with MDS or severe anemia requiring multiple blood transfusions,[32,33] and adequate iron chelation therapy is recommended for such patients.[34] The administration of oral iron-chelating agents such as deferasirox may be an attractive treatment for iron-overloaded patients compared with deferoxamine, which requires s.c. or i.v.

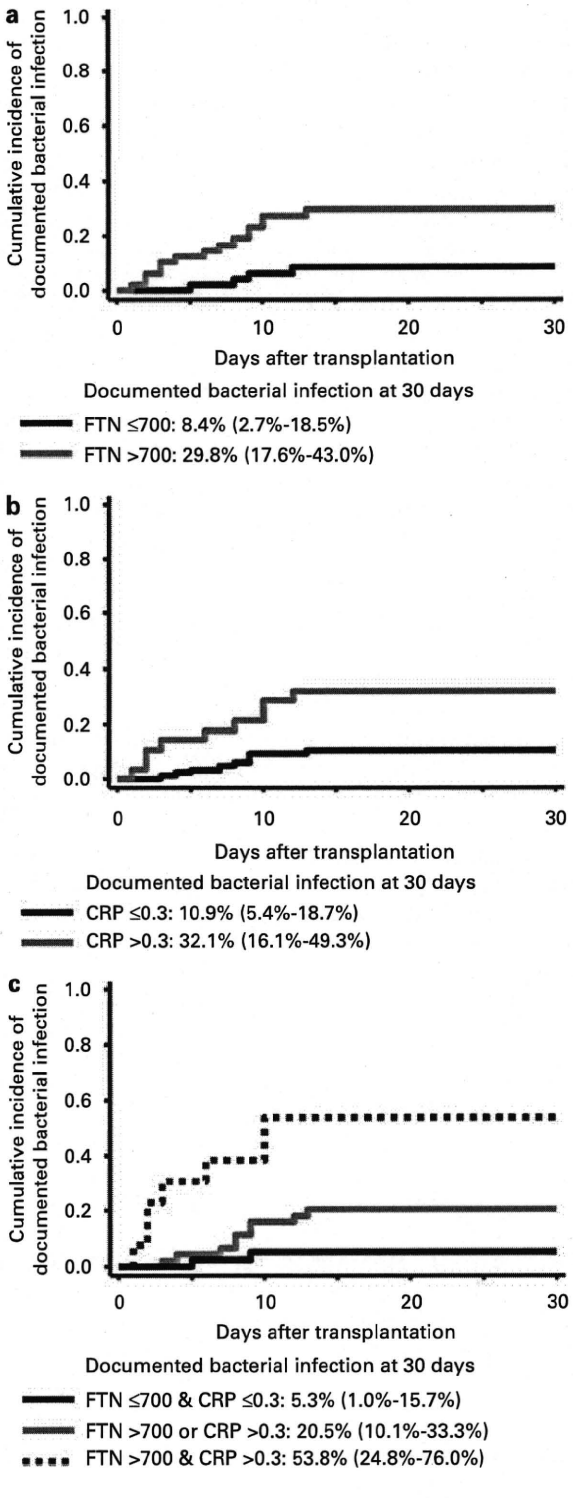

Figure 1 Cumulative incidence of documented bacterial infection within 30 days after transplantation. (**a**) Solid black line, patients with low ferritin levels (≤700 ng/mL) ($n=49$); gray line, patients with high ferritin levels (>700 ng/mL) ($n=47$), (**b**) Solid black line, patients with low CRP levels (≤0.3 mg/dL) ($n=84$); gray line, patients with high CRP levels (>0.3 mg/dL) ($n=28$), (**c**) Solid black line, patients with low ferritin (≤700 ng/mL) and low CRP levels (≤0.3 mg/dL) ($n=39$); gray line, patients with low ferritin and high CRP levels (>0.3 mg/dL) or high ferritin (>700 ng/mL) and low CRP levels ($n=44$); dotted black line, patients with high ferritin and high CRP levels ($n=13$).

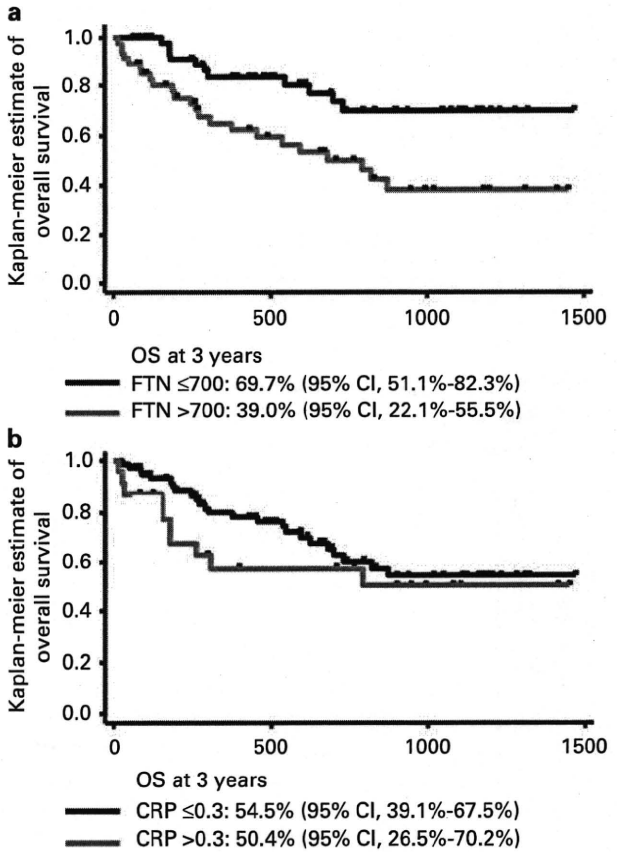

Figure 2 Kaplan–Meier estimate of overall survival after transplantation. (**a**) Solid black line, patients with low ferritin levels (≤700 ng/mL) ($n=49$); gray line, patients with high ferritin levels (>700 ng/mL) ($n=47$), (**b**) Solid black line, patients with low CRP levels (≤0.3 mg/dL) ($n=73$); gray line, patients with high CRP levels (>0.3 mg/dL) ($n=23$).

administration. However, the optimal dosage and timing for the administration of deferasirox in allogeneic HCT should be carefully determined in future studies because its renal and gastrointestinal side effects may exacerbate complications of HCT.

At present, only one report has referred to the association between pretransplant CRP levels and transplant outcomes.[18] In that report, pretransplant CRP levels had a marginally significant association with infection within 100 days after reduced-intensity HCT, whereas other confounding factors, including age, disease status, hematopoietic cell transplantation-specific comorbidity index (HCT-CI), and performance status, had no association; this result is consistent with our present findings. One possible explanation of these findings is that the slightly elevated CRP levels might have reflected minute inflammation, which may represent the presence of latent bacterial infection with negative clinical signs and negative results in pretransplant screening tests, such as X-ray or CT scans. Undetectable bacterial organisms colonized under bacteriostatic conditions before transplant might have rapidly proliferated in the post-transplant neutropenic and immunosuppressive state. Therefore, even if no bacterial infection is detected before transplant in screening tests, latent bacterial infection should be considered in patients with high CRP levels. With regard to treatment-related

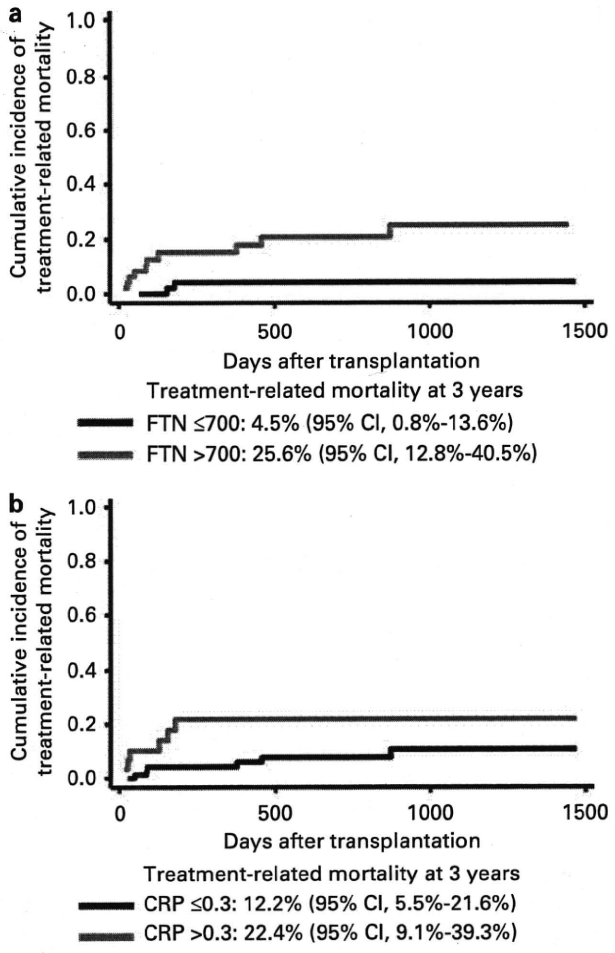

Figure 3 Cumulative incidence of treatment-related mortality after transplantation. (**a**) Solid black line, patients with low ferritin levels (≤700 ng/mL) ($n=49$); gray line, patients with high ferritin levels (>700 ng/mL) ($n=47$), (**b**) Solid black line, patients with low CRP levels (≤0.3 mg/dL) ($n=73$); gray line, patients with high CRP levels (>0.3 mg/dL) ($n=23$).

Table 4 Causes of death

Category	*Low ferritin group (≤700 ng/mL)* (n = 49)	*High ferritin group (>700 ng/mL)* (n = 47)
Within 100 days after transplant		
Infection	0	2 (29%)
Organ failure	0	2 (29%)
Graft-versus-host disease	0	2 (29%)
Hepatic veno-occlusive disease	0	1 (14%)
Total	0	7
More than 100 days after transplant		
Relapse	9 (82%)	12 (75%)
Infection	1 (9%)	2 (13%)
Organ failure	1 (9%)	0
Idiopathic pneumonia syndrome	0	1 (6%)
Bleeding	0	1 (6%)
Total	11	16

mortality, an elevated pretransplant CRP level was found to be a significant risk factor in our study, consistent with a previous report.[18] The reason for the worse treatment-related mortality in patients with elevated pretransplant CRP levels remains unclear and needs to be clarified in future studies.

To ensure the safety of allogeneic HCT with the limited use of antibacterial agents, the selective prophylactic administration of antibacterial agents such as FQs only to patients at high risk of bacterial infection may be effective. In this study, Gram-negative bacilli that were highly sensitive to FQs (93.6%) were the main bacterial organisms isolated, which suggests that these infections may have been prevented by the prophylactic administration of FQs in our center. However, this approach may be effective only if most of the bacterial isolates at the transplant center were sufficiently sensitive to these prophylactic antibiotics. In future studies, it would be worthwhile evaluating whether the incidence of early bacterial infection can be reduced by the prophylactic administration of antibiotics in patients with predefined risk factors such as high ferritin levels or high CRP levels. Iron chelation therapy before HCT is another intriguing strategy that is worthy of future evaluation.

This study had several limitations. The retrospective study design, small sample size and heterogeneous background of diseases and transplantation procedures may have biased the results. In addition, HCT-CI, including the performance status, was not evaluated in this cohort due to a lack of adequate information. Furthermore, the impact of serum ferritin levels on the outcomes should be interpreted with caution. Although we consistently determined that high ferritin levels have an adverse impact on early bacterial infection regardless of CRP levels, serum ferritin levels can be affected by conditions associated with other diseases.[35] In a future study, it may be worthwhile to quantify iron overload by other methods, such as magnetic resonance imaging of the liver,[36] and to re-analyze the effect of iron content on the outcome.

In conclusion, these results suggest that pretransplant serum ferritin and CRP levels, which can be easily measured in various centers, may be useful markers for predicting the risk of early bacterial complications after allogeneic HCT. However, larger prospective studies are warranted to validate our findings and further research is needed to identify other biomarkers that may be associated with the development of post-transplant bacterial complications.

Conflict of interest

The authors declare no conflict of interest.

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Scientific Research

Historical Cohort Study of the Efficacy and Safety of Piperacillin/Tazobactam Versus Fourth-Generation Cephalosporins for Empirical Treatment of Febrile Neutropenia in Patients with Hematological Malignancies

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ABSTRACT

We retrospectively evaluated the efficacy and safety of the combination drug piperacillin/tazobactam (PIPC/TAZ) *in comparison with those of fourth-generation cephalosporins* (4*th Cephs*) *as initial empirical treatment in hematological malignancies patients with febrile neutropenia* (*FN*). *Among* 200 *patients assessed in this study,* 49 *had received PIPC/TAZ and* 151 4*th Cephs. Patient background characteristics were comparable between the two treatment groups. The overall efficacy rate in those receiving 4th Cephs and PIPC/TAZ was* 57.0% (86/151 *patients*) *and* 59.2% (29/49 *patients), respectively, with no significant difference detected between the two treatment regimens* (P = 0.78). *Treatment did not need to be discontinued or interrupted due to development of adverse drug reactions in any of the patients. Therefore in this study the efficacy and safety of PIPC/TAZ as initial antimicrobial treatment for FN in patients with hematological malignancies were not inferior to those of* 4*th Cephs. Based on the preliminary data obtained in this study, we propose to conduct a multicenter, prospective, controlled study to compare PIPC/TAZ versus CFPM given as empirical antimicrobial treatment against FN in patients with hematological malignancies.*

Keywords: *Febrile Neutropenia, Piperacillin/Tazobactam, Fourth-Generation Cephalosporins, Safety, Efficacy*

1. Introduction

Hematological malignancies such as acute leukemia, myelodysplastic syndromes (MDS), malignant lymphoma, and multiple myeloma are often complicated by fever associated with decreases of neutrophil counts caused by anticancer drug treatment. As such, febrile neutropenia (FN) requires prompt treatment with broad-spectrum antimicrobials since it may be associated with life-threatening infections.

The Infectious Diseases Society of America (IDSA) recommends as initial treatment in patients with FN who are at high risk of serious infections either monotherapy with a third-generation cephalosporins, a fourth Generation cephalosporins (4 th Ceph; cefepime [CFPM]), or a carbapenem or dual therapy with an aminoglycoside plus an antipseudomonal penicillin (such as in the combination drug piperacillin/tazobactam; PIPC/TAZ), CFPM, ceftazidime, or carbapenem [1]. The 2007 National Comprehensive Cancer Network (NCCN) Prevention and Treatment of Cancer-Related Infections in Clinical Practice Guidelines in Oncology [2] recommend PIPC/TAZ and place the same emphasis on monotherapy with a third – or fourth-generation cephalosporins (ceftazidime or CFPM) or a carbapenem (imipenem/cilastin or meropenem) as the IDSA guidelines. The Japanese [3] and German [4] guidelines are also mostly consistent with the

IDSA guidelines.

The efficacy and safety of PIPC/TAZ given as initial treatment against FN have so far not been reported in Japanese patients. Therefore in this historical cohort study we evaluated and compared the efficacy and safety of PIPC/TAZ with those of 4th Cephs in the setting of initial antimicrobial treatment for FN, as a preliminary step to our conducting a future controlled study investigating the usefulness of PIPC/TAZ in patients with FN secondary to hematological malignancies.

2. Patients and Methods

2.1. Patients

Patients with hematological malignancies who were admitted to the Department of Hematology and Oncology at Kyoto University Hospital between January 2005 and July 2006, treated with anticancer drugs and/or transplantation, and subsequently administered either a 4th Cephs (CFPM or cefozopran) or PIPC/TAZ as initial treatment against FN were included in this study. Treatment was given on an inpatient basis. Before initiation of antibiotic therapy, blood samples for cell cultures were obtained from a peripheral vein in the context of fever and/or other signs consistent with infection. Data on specific site infections were not collected. Prophylactic antimicrobials, including quinolones, had not been used in any of the patients. This study was approved by the Ethics Committee of Kyoto University Graduate School and the Faculty of Medicine.

2.2. Antimicrobial Treatment

The daily dosage of the 4th Cephs was 4 g, with the drug administered in three divided doses (1 g each at 09:00 and 15:00; 2 g at 21:00), and that of PIPC/TAZ was 13.5 g also administered intravenously in three divided doses (PIPC 4 g/TAZ 500 mg each at 9:00, 15:00, and 21:00). The management after empiric antibiotic therapy was conducted according to the algorithm recommended by the IDSA guidelines [1]. An aminoglycoside was used concomitantly in both treatment groups at the discretion of the attending hematologists. None of the patients required adjustment of the antimicrobial drug dose or the dosing interval on account of renal dysfunction.

2.3. Study Parameters

Data for the analysis included age, sex, underlying disease, type of transplant, type of initial treatment against FN, baseline neutrophil count (at the start of initial treatment), treatment switch, and duration of neutrophil count $< 100/mm^3$ or $< 500/mm^3$ in each patient.

FN was defined as an axillary temperature $\geq 37.5°C$ with a neutrophil count $< 500/mm^3$. Thermometry was performed ≥ 3 times daily: in the morning and afternoon and before going to bed, according to the condition of each individual patient. Potential noninfectious causes of fever were not ruled out in this study. Patients treated successfully with the initial treatment alone were defined as "responders" whereas the remainder comprised the "nonresponders" group. That is, efficacy of the study drugs was assessed based on whether symptoms of FN were resolved by the initial empiric therapy or the patients were switched to other antimicrobial agents.

All adverse drug reactions were recorded on central database.

2.4. Statistical Analysis

Student's *t*-test was used to analyze the influence of age and duration of neutrophil suppression. Chi-square test or Fisher's exact test was used to analyze the influence of sex, underlying disease, and type of transplant and efficacy rate.

3. Results

3.1. Patient Characteristics

Of the 200 patients included in this study, 151 were treated with 4th Cephs and 49 received PIPC/TAZ (P/T group) as initial empirical treatment against FN. None of the patients died during the study, which was conducted for ≤ 30 days of treatment. The patient characteristics are presented in **Table 1**. Seventy-eight patients in the 4th Cephs group (52%) and 30 patients in the P/T group (55%) had acute leukemia or MDS ($P = 0.67$). Twenty-two patients in the 4th Cephs group (15%) and 8 patients in the P/T group (16%) had undergone transplantation of hematopoietic progenitor cells ($P = 0.76$). All patients in both groups had neutrophil counts $< 500/mm^3$ at the start of treatment. Furthermore, 45 patients in the 4th Cephs group (30%) and 14 patients in the P/T group (29%) had neutrophil counts $< 100/mm^3$ at the start of treatment ($P = 0.87$). The mean duration of having a neutrophil count $< 100/mm^3$ and $< 500/mm^3$ was comparable between the two treatment groups ($P = 0.23$ and 0.60, respectively). Forty-two patients in the 4th Cephs group (28%) and 12 patients in the P/T group (24%) were treated concurrently with an aminoglycoside ($P = 0.65$). Etiologic organisms obtained from blood culture examinations were positively identified in 11 patients in the 4th Cephs group (*Escherichia coli*, n = 3; *Klebsiella pneumoniae*, n = 3; *Pseudomonas aeruginosa*, n = 2; *Enterobacter cloacae*, n = 1; *Klebsiella oxytoca*, n = 1; *Streptococcus mitis*, n = 1) and in four patients in the P/T group (*E. coli*, n = 3; *Streptococcus viridans*, n = 1).

Table 1. Baseline characteristics of patients with febrile neutropenia.

Parameter	4th Cephs group (n = 151)	P/T group (n = 49)	P-value
Mean age (range), years	52.1(18–81)	52.8(24–74)	0.77
Sex, M/F	67/84	25/24	0.42
Underlying disease, n (%)			
Acute leukemia and MDS	78 (52)	30 (61)	0.24
Malignant lymphoma	46 (30)	15 (22)	0.98
Multiple myeloma	14 (9)	2 (4)	0.37
Chronic leukemia	10 (7)	0	0.12
Immunoblastic lymphadenopathy	2 (1)	1 (2)	0.57
Aplastic aplasia	1 (1)	0	0.99
Plasmacytoma	0	1 (1)	0.25
Transplantation, n (%)	22 (15)	8 (16)	0.76
Myeloablative	10 (7)	4 (8)	0.75
Nonmyeloablative	8 (5)	2 (4)	0.99
Autologous	4 (3)	2 (4)	0.64
Neutrophil count, n (%)			
< 100/mm^3	45 (30)	14 (29)	0.87
100–500/mm^3	106 (70)	35 (71)	0.87
Mean (SD) duration of neutropenia, days			
< 100/mm^3	9.9 (8.3)	11.9 (9.5)	0.23
< 500/mm^3	12.6 (10.5)	13.5 (10.3)	0.60
Dual therapy, n (%)	42 (28)	12 (24)	0.65

4th Cephs: fourth-generation cephalosporins, P/T: piperacillin/tazobactam.

Table 2. Efficacy rate of fourth-generation cephalosporins or piperacillin/tazobactam as initial empirical therapy in patients by subgroup.

	Efficacy rate, n (%)		P-value
	4th Cephs group (n = 151)	P/T group (n = 49)	
Total	86/151 (57.0)	29/49 (59.2)	0.78
Underlying disease			
Acute leukemia and MDS	39/78 (50.0)	17/30 (56.7)	0.53
Other hematological disorders	47/73 (64.4)	12/19 (63.2)	0.92
Transplantation			
Yes	9/22 (40.9)	4/8 (50.0)	0.70
No	77/129 (59.7)	25/41 (61.0)	0.88
Monotherapy	65/109 (59.6)	22/37 (59.5)	0.99
Dual therapy	21/42 (50.0)	7/12 (58.3)	0.61

4th Cephs: fourth-generation cephalosporins, P/T: piperacillin/tazobactam.

3.2. Clinical Efficacy

Efficacy rates of the two test agents given as initial empiric therapy against FN are presented in **Table 2**. In the 4th Cephs and P/T groups, the overall efficacy rate was 57.0% (86/151 patients) and 59.2% (29/49 patients), respectively, with no significant difference detected between the two groups (P = 0.78). Moreover, the difference of efficacy rate was not statistically significant between acute leukemia and MDS patients in the 4th Cephs group (50.0% [39/78 patients]) and P/T group (56.7% [17/30 patients]; P = 0.54) and in those with other hematological disorders (64.4% [47/73 patients] and 63.2% [12/19 patients], respectively; P = 0.92). Furthermore, the between-group efficacy rate was not different in posttransplant patients (40.9% [9/22 patients] and 50.0% [4/8 patients], respectively; P = 0.70) and those without transplantation (59.7% [77/129 patients] and 61.0% [25/41 patients], respectively; P = 0.88). The efficacy rate in patients receiving monotherapy was 59.6% (65/109) in the 4th Cephs group and 59.5% (22/37 patients) in the P/T group (P = 0.99) whereas in those concomitantly receiving an aminoglycoside the rate was 50.0% (21/42 patients) and 58.3% (7/12 patients), re-

spectively ($P = 0.61$).

3.3. Adverse Drug Reactions

No adverse drug reaction requiring discontinuation or switching of the study treatments was noted in any of the patients.

4. Discussion

According to the Japan Adult Leukemia Study Group's Supportive Therapy Subcommittee questionnaire survey of 196 participating institutions nationwide, cephalosporin ± aminoglycoside, carbapenem ± aminoglycoside, and antipseudomonal penicillin ± aminoglycoside were used as the initial treatment for FN in 51%, 23%, and 11% of the responding institutions, respectively [5]. The 2002 IDSA guidelines and recently published Japanese guidelines for antimicrobial therapy against FN recommend monotherapy with a broad-spectrum cephalosporin or carbapenem, combination of both these drug classes, or antipseudomonal penicillin and an aminoglycoside [1,3]. Increasing emergence of multidrug-resistant *Pseudomonas* has become a clinical problem in recent years [6-10]. Concomitant use of carbapenems with cancer chemotherapy is a potential risk factor for the development of multidrug-resistant *Pseudomonas* infection [11]. We consider PIPC/TAZ as an alternative to carbapenems because of these two medications' comparable antimicrobial spectrums.

In the present study, the overall efficacy rate was 57.0% in the 4th Cephs group and 59.2% in the P/T group. Similar to our findings, the efficacy rates of 4th Cephs ± aminoglycoside and PIPC/TAZ ± aminoglycoside in cases of FN reported from previous controlled studies varied at 21–62% and 27%–61%, respectively [12-15]. This variability in the efficacy rates noted in these studies is likely related to differences in the definition of efficacy, which was variously set as 2– or 3-day defervescence, microbiological eradication, test of cure, and so on.

Precise indications for the concurrent use of aminoglycosides in the initial treatment against FN remain controversial. There is no reference to this issue in the IDSA guidelines, while the Japanese guidelines recommend concurrent use of an aminoglycoside as an option in patients receiving induction therapy for acute leukemia and those undergoing hematopoietic stem cell transplantation [1,3]. As in previous studies, 20-25% of patients included in the present study received concurrent administration of an aminoglycoside with one of the guideline-recommended drugs as initial treatment. One previous study reported a higher efficacy rate in patients receiving adjuvant aminoglycoside than in those receiving monotherapy [16]. In the present study, on the other hand, the efficacy rate in patients receiving concurrent aminoglycoside was slightly lower compared with that in patients on monotherapy in both treatment groups, although the difference was not statistically significant ($P = 0.29$ and 0.95 in the 4th Cephs and P/T groups, respectively). Use of dual therapy in many posttransplant patients and in patients with acute leukemia/MDS may be the reason for the lower efficacy rate associated with concurrent aminoglycoside administration in our study.

One of the main objectives of this study was to determine whether the efficacy of PIPC/TAZ is comparable to that of 4th Cephs as initial treatment against FN, as a preliminary assessment of the feasibility of our conducting a future prospective controlled study in this setting. The main limitations of this study were its non-prospective design and the efficacy evaluation being not solely based on fever reduction as in previous Japanese studies [16,17]. Here, treatment efficacy was evaluated based on the need for switching antimicrobial drugs, because this was determined by attending hematologists on the basis of comprehensive assessments of clinical symptoms, laboratory tests, and radiological findings in individual patients. The third limitation of this study is that the antimicrobials were given to the patients at 9:00, 15:00, and 21:00 hours, taking into account the patients' sleeping times and the nursing shifts. As reported elsewhere, intravenous antimicrobial infusion should desirably be given at 8-hour intervals according to the antimicrobial pharmacokinetics/pharmacodynamics [12-15].

We found that the efficacy and safety of PIPC/TAZ given as initial treatment against FN were not inferior to those of 4th Cephs. Based on the preliminary data obtained in this study, we propose to conduct a multicenter, prospective, controlled study to compare PIPC/TAZ versus CFPM given as empirical antimicrobial treatment against FN in patients with hematological malignancies.

This study was presented, in part, at the 54th Japanese Society of Chemotherapy West Japan Branch Conference and was awarded the 1st Japanese Society of Chemotherapy Head of West Japan Branch Award (Clinical Division).

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ORIGINAL ARTICLE

Separation of antileukemic effects from graft-versus-host disease in MHC-haploidentical murine bone marrow transplantation: participation of host immune cells

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Abstract Allogeneic hematopoietic stem cell transplantation (HSCT) is associated with both graft-versus-host disease (GVHD) and graft-versus-leukemia (GVL) effects. In clinical studies of HLA-mismatched HSCT, strong GVL effects have been reported. In the present study, we addressed the mechanism of the GVL and GVH response using MHC-haploidentical murine bone marrow transplantation (BMT) models. Recipient BDF1 (H-$2^{b/d}$) mice received T cell-depleted bone marrow and spleen cells from B6C3F1 (H-$2^{b/k}$) or C57BL/6 (H-2^{b}) mice with or without P815 mastocytoma cells (H-2^{d}) after receiving lethal total body irradiation. B6C3F1 → BDF1 (hetero-to-hetero type) recipients showed more powerful antileukemic effects with less severe GVHD than C57BL/6 → BDF1 (parent-to-F1 type) recipients. Compared with C57BL/6 → BDF1 recipients, significantly higher in vitro cytotoxic activity against P815 cells was observed in B6C3F1 → BDF1 recipients. Significantly lower CXCR3 expression on donor T cells and higher interferon (IFN)-γ expression were considered to be associated with strong antileukemic effects with less severe GVHD in B6C3F1 → BDF1 recipients. Furthermore, host immune cells, especially natural killer cells and $CD8^{+}$ T cells, were found to contribute remarkably to high IFN-γ production in B6C3F1 → BDF1 recipients. Thus, in MHC-haploidentical HSCT, host immune cells may change the balance between GVH and GVL response through IFN-γ production.

Keywords MHC-mismatched hematopoietic stem cell transplantation · GVHD · GVL · Interferon-γ · Natural killer cell

1 Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) has been a potentially curative therapy for patients with a variety of diseases, especially for hematologic malignancies [1, 2]; however, more than 70% of patients who could benefit from allogeneic bone marrow transplantation (BMT) do not have a matched sibling donor. On the other hand, there is a greater than 90% chance of promptly identifying a human leukocyte antigen (HLA)-haploidentical donor within the family. A major obstacle of HLA-mismatched HSCT is the high incidence of graft-versus-host disease (GVHD) [3, 4]; therefore, separating beneficial GVL effects from deleterious GVHD is a goal for HLA-mismatched HSCT.

In this context, we have reported, in a series of clinical studies on unmanipulated HLA-haploidentical HSCT, that strong graft-versus-leukemia (GVL) effects are maintained in many patients even after complete suppression of GVHD by the use of reduced-intensity conditioning treatment, or the use of steroids and/or anti-T-lymphocyte globulin as

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GVHD prophylaxis [5–8]. However, the cellular and molecular mechanisms of the separation of GVL reaction from GVHD observed in HLA-haploidentical HSCT remain unclear. In murine BMT studies, parent-to-F1 (homo-to-hetero) BMTs, in which donor type engraftment can be achieved without total body irradiation (TBI), have usually been used as major histocompatibility complex (MHC)-haploidentical BMT models. Furthermore, these models have contributed to the progress of GVHD study [9] because the influence of radiation on tissue damage can be avoided; however, whether the parent-to-F1 murine models correctly reflect clinical HLA-haploidentical HSCTs that are mostly performed in transplant settings of HLA hetero-to-hetero combinations remains unclear.

To enable comparison between homo-to-hetero and hetero-to-hetero transplants, we therefore established two major MHC-haploidentical murine BMT models, in which recipient BDF1 (H-$2^{b/d}$) mice received T cell-depleted (TCD) bone marrow (BM) and spleen cells from B6C3F1 (H-$2^{b/k}$) or C57BL/6 (H-2^{b}) mice with or without P815 mastocytoma cells after receiving lethal TBI. In the present study, we found that B6C3F1 → BDF1 (MHC hetero-to-hetero-type) BMT showed more powerful antileukemic effects with less severe GVHD than C57BL/6 → BDF1 (MHC homo-to-hetero-type) BMT. Furthermore, we found that, compared with C57BL/6 → BDF1 recipients, B6C3F1 → BDF1 recipients showed lower CXCR3 expression on donor T cells in recipient spleens and higher interferon (IFN)-γ production. This high IFN-γ milieu with low expression of the inflammatory chemokine receptor was considered to be associated with the induction of strong antileukemic effects with less severe GVHD, since recent studies demonstrated that IFN-γ augmented lymphohematopoietic GVH reactions [10–12], namely, GVL reaction, and that IFN-γ mediated the protective effect against GVHD [13, 14]. Furthermore, donor immune cells as well as host immune cells, especially host natural killer (NK) cells and $CD8^{+}$ T cells, were found to home to spleens after transplantation, and to produce IFN-γ highly in B6C3F1 → BDF1 recipients.

2 Materials and methods

2.1 Mice

Female C57BL/6 (B6, H-2^{b}), B6C3F1 (B6 × C3H/HeJ; H-$2^{b/k}$) or BDF1 (B6 × DBA2; H-$2^{b/d}$) mice were purchased from Japan CLEA (Osaka, Japan), or Shizuoka Laboratory Animal Center (Shizuoka, Japan). Mice used for experiments were 8–12 weeks of age, were housed in sterile microisolator cages in a specific pathogen-free mouse facility, and received autoclaved food and water ad libitum.

2.2 BMT

BM cells were harvested from the tibia and femur of donor mice by flushing with RPMI-1640 medium. T cell depletion of BM cells was performed by treatment with anti-Thy1.2 monoclonal antibody (mAb) (clone 30-H-12; PharMingen, San Diego, CA, USA) plus rabbit complement (Cedarlane, Hornby, ON, Canada). Spleen cells were isolated from donor mice using the nylon-wool-purification method as a source of lymphocytes. All BMTs were performed by the transfusion of a fixed number of donor cells after TBI the previous day. TBI was given in a single dose at a dose rate of 50 cGy/min. Cells from donors were resuspended in 0.5 ml RPMI-1640 medium and transplanted by tail-vein infusion into recipients.

Survival was monitored daily, and the presence of GVHD was judged by clinical symptoms, including body weight, posture (hunching), mobility, fur texture, and skin integrity [15]. All animal protocols were approved by the Ethics Review Committee for Animal Experimentation of Hyogo College of Medicine.

2.3 Challenge of tumor cells

In experiments to estimate the strength of antileukemic effects, recipient mice received P815 mastocytoma cells derived from DBA/2 (H-2^{d}). The tumor cells were injected intravenously through the tail vein on the day of transplantation.

2.4 Histopathological analysis

Tissues were fixed in 10% buffered formalin and embedded in paraffin. The sections were stained with hematoxylin and eosin and were examined by light microscopy. Immunohistochemical analysis was performed as previously described [16], with some modifications. In brief, frozen sections were fixed in 4% paraformaldehyde. After being blocked with phosphate-buffered saline (PBS) containing 10% fetal calf serum (FCS) for 15 min at room temperature, the origin of infiltrating T cells was determined by staining with mouse anti-H-$2K^{d}$ mAb (SF1-1.1; host-specific) and rat anti-CD4 mAb (GK1.5) or rat anti-CD8 mAb (H35-17.2) at 4°C overnight and visualized using Alexa-Fluor 488-labeled anti-rat and Alexa-Fluor 546-labeled anti-mouse antibody. 4′,6-Diamidino-2-phenylindole (DAPI) was used to stain the nucleus. Sections for fluorescent staining were analyzed with a confocal laser scanning microscope (LSM510; Carl Zeiss, Jena, Germany) [16].

2.5 In vivo spectral fluorescence imaging analysis

For in vivo imaging analysis, P815 cells were engineered to express mCherry fluorescent protein by a lentiviral vector

transduction system, as previously described [17, 18]. Recipient mice received modified P815 cells with BM and spleen cells via the tail vein on day 0. Prior to imaging, mice were anesthetized with sodium pentbarbital (Nembutal), and hair was removed with a hair removal cream, Epilat (Kracie, Tokyo, Japan), and rinsed with water. Spectral fluorescence imaging analysis was performed using the Maestro in vivo fluorescence imaging system (CRi; Woburn, MA, USA), as previously described [19]. Whole body images (0.05- to 0.5-s exposure) were taken and analyzed over sequential days.

2.6 Flow cytometric analysis

Anti-Fc receptor (2.4G2) monoclonal antibody (mAb), fluorescein isothiocyanate (FITC)-conjugated anti-mouse H-2K^d (clone SF1–1.1) mAb, phycoerythrin (PE)- indotricarbocyanine (Cy7)-conjugated anti-mouse CD3 (clone 145-2C11) mAb, anti-mouse CD4 (clone GK1.5) mAb, allophycocyanin (APC)-conjugated anti-mouse CD8 (clone 53–6.7) mAb, and PE-conjugated anti-mouse NK1.1 (clone PK136) mAb were purchased from PharMingen (San Diego, CA, USA). PE-conjugated anti-mouse CXCR3 (clone 220803) mAb and rat anti-mouse IgG_{2A} isotype control were purchased from R&D Systems (Minneapolis, MN, USA). Cell suspensions were prepared in PBS-containing 1% FCS and 0.1% sodium azide. Cells were incubated with an anti-Fc receptor mAb for 10 min at 4°C to block nonspecific staining and then incubated with FITC-, PE-Cy7-, APC-, and PE-conjugated mAb for 30 min. The stained cells were washed twice, resuspended, and analyzed using FACSCalibur (Becton–Dickinson, Mountain View, CA, USA) using CellQuest software (Becton–Dickinson).

Intracellular IFN-γ staining was performed using the BD Cytofix/Cytoperm^{TM} Fixation/Permeabilization kit (BD Bioscience, San Jose, CA, USA). In brief, cells were retrieved from the recipient spleen, and resuspended at 10^6/ml and cultured with phorbol myristic acid at 50 ng/ml plus ionomycin at 500 ng/ml for 5 h, including monensin during the last 2 h of culture. Cells were harvested, washed, and resuspended in PBS-containing 1% FCS and 0.1% sodium azide. Cell-surface antigens were then stained as described above, and cells were resuspended in 100 µl per well of a microwell plate of fixation/permeabilization solution, and incubated for 20 min at 4°C. After washing, cells were stained with APC-conjugated anti-IFN-γ (clone XMG1.2; PharMingen) or isotype control: rat IgG1-APC (Clone R3-34).

2.7 Mixed lymphocyte culture (MLC) and ^{51}Cr release assay

BDF1 mice were transplanted using TCD-BM (5×10^6) and spleen cells (2×10^7) after receiving TBI 9 Gy. Spleen cells of the recipient mice on day 14 were used as responders for MLC. Cells (3×10^5/200 µl/well) were cultured with irradiated (20 Gy) BDF1 spleen cells (3×10^5/200 µl/well) in 24-well flat-bottomed plates (Falcon Labware, Lincoln Park, NJ, USA). After 72 h culture, IFN-γ concentrations of the culture supernatants were measured by Bio-Plex (Bio-Rad Laboratories, Hercules, CA, USA). For cytotoxic T lymphocyte (CTL) assay, BDF1 mice were transplanted using TCD-BM (5×10^6) and spleen cells (2×10^7) with P815 cells (1×10^4) after receiving TBI 9 Gy. Spleen cells of the recipient mice on day 14 were recovered, and directly measured for CTL activity against P815 cells by ^{51}Cr release assay, as described elsewhere [20]. Effecter cells were tested in triplicate at four effector:target (E:T) ratios, and the percent lysis was calculated according to the following formula: [(sample cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm)] × 100%. Results are shown as the mean percent lysis of the E:T cell ratio for each treatment group.

2.8 Statistical analysis

Values were compared by two-tailed Student's t test. Survival data were plotted by the Kaplan–Meier method and were analyzed by the log-rank test. A P value of less than 0.05 was considered significant.

3 Results

3.1 B6C3F1 → BDF1 recipients showed less severe GVHD than C57BL/6 → BDF1 recipients

To investigate the pathophysiology of GVH or GVL reactions in MHC-haploidentical BMT, we established 2 MHC-haploidentical murine BMT models: BDF1 (H-2$^{b/d}$) mice were transplanted from B6C3F1 (H-2$^{b/k}$) or C57BL/6 (H-2^b) mice. B6C3F1 → BDF1 is an MHC hetero-to-hetero (donor/recipient combination) BMT model, where one MHC haplotype is identical between the donor and recipient but the other is different. C57BL/6 → BDF1 is an MHC homo-to-hetero (parent-to-F1) BMT model, where MHC is haplotypically mismatched in the graft-versus-host (GVH) direction but not in the host-versus-graft (HVG) direction.

Recipient BDF1 mice received donor TCD-BM (5×10^6) and spleen (2×10^7) cells after a lethal TBI dose (9 Gy) the previous day. There was no significant difference in total cell numbers, T cell doses, and the CD4:CD8 ratio of spleen cells transfused between the 2 BMT models (data not shown). Two weeks after BMT, the majority of C57BL/6 → BDF1 recipients began to present

GVHD signs, such as body weight loss and a hunching posture, and 90% of mice had died of GVHD by day 40 (Fig. 1a). In contrast, B6C3F1 → BDF1 recipients showed fewer GVHD signs, and only 30% of mice had died of GVHD by day 80, with significantly improved survival observed in B6C3F1 → BDF1 recipients compared with C57BL/6 → BDF1 recipients. Histopathological examination of C57BL/6 → BDF1 recipients on day 14 revealed prominent lymphocyte infiltration in the periportal area of the liver, and various pathological changes in the large intestine compatible to GVHD (Fig. 1b, left panel). In the immunohistochemical study, these lymphocytes infiltrating the liver or large intestine were found to be donor-derived CD4 or CD8 T cells (Fig. 1c, upper panel). In contrast, liver or large intestine samples from B6C3F1 → BDF1 recipients showed few pathological changes (Fig. 1b, right panel) with almost no infiltration of donor T cells (Fig. 1c, lower panel). These results indicate that B6C3F1 → BDF1 recipients developed less severe GVHD than C57BL/6 → BDF1 recipients, leading to improved survival in B6C3F1 → BDF1 recipients.

3.2 B6C3F1 → BDF1 recipients induced more powerful antileukemic effects than C57BL/6 → BDF1 recipients

To compare antileukemic effects in the 2 MHC-haploidentical BMTs, recipient BDF1 mice received P815 mastocytoma cells (H-2^d, 1×10^4) with donor TCD-BM cells (5×10^6) with or without donor spleen cells (2×10^7) after receiving TBI 9 Gy the previous day. In mice receiving TCD-BM cells alone, P815 cells proliferated mainly in the liver, spleen, and BM of the recipient, and tended to form macroscopic nodules in the liver or spleen. Some animals developed lower limb paralysis, and histological analysis revealed infiltration of P815 cells around the spinal cord. Thus, death of recipient mice accompanied by these signs or symptoms was considered leukemic death. When recipient mice presenting with clinical signs of GVHD died without any signs of leukemia progression, they were considered as death by GVHD. All mice receiving TCD-BM cells alone with P815 cells had died of leukemia progression by day 20 (Fig. 2a). Compared with mice receiving TCD-BM cells alone, mice receiving spleen cells showed a significantly improved survival in the 2 groups (Fig. 2a); however, none of them died of tumor progression (some mice died of GVHD). We could demonstrated antileukemic effects of donor spleen cells, but could not compare antileukemic effects in the 2 BMT models under these conditions.

Fig. 1 Survival and histological change in B6C3F1 → BDF1 and C57BL/6 → BDF1 recipients. **a.** Survival of B6C3F1 → BDF1 and C57BL/6 → BDF1 BMT recipients. All recipients receiving TCD-BM cells alone from C57BL/6 or B6C3F1 mice survived. For mice receiving TCD-BM and spleen cells, B6C3F1 → BDF1 mice showed significantly improved survival than C57BL/6 → BDF1 mice. All mice that died showed severe clinical signs of GVHD. *Open rectangles* C57BL/6 → BDF1 mice receiving TCD-BM cells only ($n = 4$); *open triangles* B6C3F1 → BDF1 mice receiving TCD-BM cells alone ($n = 4$), *closed rectangles* C57BL/6 → BDF1 mice receiving TCD-BM and spleen cells ($n = 10$), *closed triangles* B6C3F1 → BDF1 mice receiving TCD-BM and spleen cells ($n = 10$). $^{**}P$ value < 0.01. The results are representative of 2 separate experiments. **b** Histological analysis of the liver and large intestine from recipient mice receiving TCD-BM and spleen cells. Prominent lymphocyte infiltration in the periportal area of the liver and severe intestinal histopathological changes, including surface erosion, decreased numbers of goblet cells, and cellular infiltration in the lamina propria, were observed in samples from C57BL/6 → BDF1 recipients on day 14. In contrast, few pathological changes were observed in samples from B6C3F1 → BDF1 recipients. Representative data are shown (×200). **c** Immunohistochemical analysis of GVHD-target organs on day 12. Data represent multicolored immunofluorescent staining: anti-CD4 (*green*) or anti-CD8 (*green*), anti-H2Kd (host-specific; *red*) and DAPI staining (*blue*) of the nucleus. Donor and host T cells were visualized as *green* and *yellow*, respectively. Massive lymphocytes infiltrating the liver or large intestine in C57BL/6 → BDF1 recipients were found to be donor-derived CD4 or CD8 T cells. In contrast, fewer infiltrates of donor T cells into these organs were observed in B6C3F1 → BDF1 recipients (×300)

Therefore, we decreased the number of spleen cells transfused to 5×10^5 cells. At this spleen cell dose, no mice died of GVHD. All mice receiving TCD-BM cells alone had died of tumor progression by day 14. Recipients receiving spleen cells survived significantly longer than mice receiving TCD-BM cells alone. All of C57BL/6 → BDF1 recipients receiving spleen cells had died of tumor progression by day 28, while only 20% of B6C3F1 → BDF1 recipients receiving spleen cells died of tumor progression during the observation period (Fig. 2b). For mice receiving spleen cells, compared with C57BL/6 → BDF1 recipients, B6C3F1 → BDF1 recipients showed a significant lower tumor mortality rate (Fig. 2b). To visualize the kinetics of tumor progression, P815 cells that were engineered to express mCherry fluorescent protein by a lentiviral transduction system were applied to the experiment in Fig. 2b. As shown in Fig. 2c, in mice receiving TCD-BM alone, fluorescence tumor signals appeared in the abdominal region (e.g. liver and spleen) and the femoral and sternal bones on day 10. In C57BL/6 → BDF1 recipients receiving TCD-BM and spleen cells, fluorescence tumor signals appeared in the femoral and sternal bones on day 10, and extended to the abdominal region by day 12. These fluorescence signals continued to strengthen, with signals continuing to spread