red blood cells and platelets were transfused using leukocyte-depleting filters.

CMV infection was defined as isolation of CMV or detection of viral proteins or nucleic acid in any body fluid or tissue specimen. CMV disease was diagnosed as follows: CMV enterocolitis was diagnosed by gastrointestinal symptoms with histologic demonstration of CMV on biopsy materials obtained by endoscopy; CMV pneumonia was diagnosed when either a bronchoalveolar lavage or a lung biopsy was positive for CMV in a patient with characteristic signs, symptoms, and chest radiographic findings; CMV retinitis was diagnosed by characteristic retinal opacities without other likely explanations for the retinal findings. CMV pp65 antigenemia was monitored weekly after engraftment or when patients died before engraftment. Briefly, 1.5×10^5 peripheral blood leukocytes were attached to slides using a cytocentrifuge and fixed with cold acetone. From 1/3 to 1/2 of the centrifuged cells were fixed on the slides. The cells were incubated with monoclonal antibody HRP-C7 (Teijin, Tokyo, Japan) raised against immediate-early antigen, and stained by the direct immunoperoxidase method. These cells were analyzed under a light microscope and results were presented as the number of positive cells per 50,000 cells [17].

CMV antigenemia was managed according to the report by Kanda et al [17]. If CMV pp65-positive cells exceeded 10/50,000, patients preemptively received either ganciclovir 5 mg/kg once daily or foscarnet 30 mg/kg twice daily. Initiation of ganciclovir or foscarnet with <10 positive cells was optional in the patients who received more than 0.5 mg/kg of prednisolone. The doses were adjusted for renal function [18]. Ganciclovir or foscarnet was discontinued when 2 consecutive results of CMV antigen were negative. When CMV disease was diagnosed during preemptive therapy, we increased the dose of ganciclovir to 5 mg/kg twice a day, or foscarnet to 60 mg/kg twice or 3 times daily.

Endpoints and Statistical Analysis

The aims of this study were (1) to determine the incidence of CMV infection after RI-CBT, (2) to investigate its clinical features, and (3) to identify its risk factors. The cumulative incidences of CMV disease and CMV reactivation defined by the detection of CMV pp65 were evaluated using Gray's method [19], considering death without CMV reactivation as a competing risk. Potential confounding factors considered in the analysis were patient's age, sex, stem cell doses, HLA disparity, GVHD prophylaxis, conditioning regimens, and aGVHD. The influence of these factors on the incidence of CMV disease and CMV reactivation was evaluated with the proportional hazard modeling treating the development of aGVHD and the use of corticosteroids as time-dependent covariates. Factors associated with at least borderline significance ($P < .10$) in the univariate analyses were subjected to a multivariate analysis using backward stepwise proportional-hazard modeling. P-values of $<.05$ were considered statistically significant.

RESULTS

Patient's Characteristics and Clinical Outcomes

Patient's characteristics are shown in Table 1. Of the 140 RI-CBT recipients, 112 patients (80%) achieved primary engraftment on a median of day 20 (range, 10-57). Sixty (43%) and 8 (6%) patients died of transplant-related causes and disease progression, respectively, within 100 days of RI-CBT. Preengraftment immune reaction [20] was diagnosed in 67 patients. Of the 112 patients who achieved engraftment, 47 (42%) developed grade II-IV aGVHD at a median onset of day 25 (range, 13-94). Sixty-one patients received prednisolone or methylprednisolone >0.5 mg/kg/day within 100 days of RI-CBT because of preengraftment immune reaction (n = 26), engraftment syndrome (n = 3), aGVHD (n = 16), and others (n = 16). As of November 2005, the median follow-up of the surviving patients was 13.0 months (range, 1.0-40.7). Overall survival rates were 85% (95% confidence interval [CI]; 79-91%) and 53% (95% CI; 45-62%) at days 30 and 100, respectively.

Clinical Features of CMV Reactivation and Diseases

Clinical features of CMV reactivation and diseases are summarized in Table 2. CMV antigenemia was found in 77 patients (55%, 95% CI; 51-59%) on a median of day 35 (range, 4 to 92). Twenty-eight of those patients received prednisolone or methylprednisolone >0.5 mg/kg/day before development of

Table 2. *Clinical Features of CMV Reactivation and Disease*

Variable
CMV reactivation
Number of patients
Onset (median [range])
Maximal levels of CMV antigenemia (range)
Preemptive therapy (ganciclovir/foscarnet/none)
CMV disease
Number of patients
Diagnose of CMV disease (median, [range])
Organ involvement
enterocolitis
pneumonia
retinitis
adrenalitis
Use of anti-CMV agents at the onset of CMV disease (ganciclovir/foscarnet/none)

CMV indicates cytomegalovirus.

Table 3. *Univariate and Multivariate Analyses for the Incidence of CMV Reactivation*

Factor	Relative risk (95% CI)	P value
Univariate		
Age	1.005 (0.989-1.021)	.53
Sex	1.10 (0.71-1.72)	.66
Disease status	0.68 (0.44-1.07)	.098
Number of HLA mismatch	1.37 (0.80-2.36)	.25
Number of infused mononuclear cells	0.99 (0.68-1.45)	.95
Number of infused $CD34^+$ cells	1.55 (1.28-1.87)	5.8×10^{-6}
GVHD prophylaxis (cyclosporine vs. tacrolimus)	0.59 (0.37-0.94)	.025
Preengraftment immune reaction	1.14 (0.74-1.78)	.55
Acute GVHD (grade II-IV)	1.21 (0.70-2.10)	.49
Use of steroid*	1.64 (1.02-2.64)	.042
Multivariate		
Number of infused $CD34^+$ cells	1.55 (1.28-1.87)	5.8×10^{-6}

CMV indicates cytomegalovirus; CI, confidence index; GVHD, graft-versus-host disease.
*Use of prednisolone or methyl-prednisolone >0.5 mg/kg/day.

CMV antigenemia. Forty-nine patients received foscarnet (n = 41) or ganciclovir (n = 8) preemptively. Initial dose of ganciclovir was 5 mg/kg once daily. The remaining 28 patients had not received foscarnet or ganciclovir according to our preemptive strategy, because of <10/50,000 of CMV pp65-positive cells.

Diagnosis of CMV disease was established in 22 patients (16%, 95% CI; 13-19%) on a median of day 33 (range 15-106); the diagnosis comprised enterocolitis (n = 21) and adrenalitis (n = 1). Of the 22 patients, 9 patients had received preemptive therapy before developing CMV disease. The remaining 13 patients had not received foscarnet or ganciclovir according to our preemptive strategy, mostly because of <10/50,000 of CMV pp65-positive cells.

Diagnosis of CMV disease was established at postmortem examination in 1 patient. The other 21 patients were treated with either foscarnet or ganciclovir. CMV disease was successfully treated in 14 patients. The remaining 8 patients died without improvement of CMV disease, although CMV disease was not the primary cause of death in any of these 8 patients.

Risk Factors of CMV Antigenemia and CMV Disease

Risk factors of CMV reactivation and CMV disease were shown in Table 3 and Table 4, respectively. CD34-positive cell dose was significantly associated with CMV reactivation on multivariate analysis (relative risk, 1.55; 95% CI 1.28-1.87; $P = 5.8 \times 10^{-6}$). Grade II-IV aGVHD was a risk factor of CMV disease on multivariate analysis (relative risk, 3.48; 95% CI 1.47-8.23; $P = .0045$).

DISCUSSION

The present study demonstrated that CMV infection is a significant complication of RI-CBT. The incidence of CMV reactivation was 55% in our study, which was comparable with previous reports on RIST [21-23] and myeloablative bone marrow transplantation (BMT) and PBSCT [24,25]. In contrast, previously reported incidence of CMV reactivation after CBT (79%) [8] was higher than that of ours, although it is the only previous report on CMV reactivation after CBT. The differences in preparative regimens and patient characteristics between the study [8] and ours may have affected the incidence of CMV reactivation. One of the unique findings in the present study was that the timing of CMV reactivation after RI-CBT was earlier than that after RIST without in vivo or ex vivo T cell depletion [21]. Another unique finding was the high incidence of CMV disease compared with transplantation of other stem cell sources [21,24,25]. Of the 77 patients with CMV antigenemia, 22 developed CMV disease in our study. The risk of progression from CMV reactivation to CMV disease may be high in CBT because of the intense immunosuppression [26].

The present study suggests that CMV infection is more likely to reactivate and to progress in RI-CBT than in transplantation using other stem cell sources. Several reasons can explain this hypothesis. First, the preparative regimens including total body irradiation (TBI) in our study might have damaged recipient-

Table 4. *Univariate and Multivariate Analyses for the Incidence of CMV Disease*

Factor	Relative risk (95% CI)	P value
Univariate		
Age	1.021 (0.991-1.052)	.16
Sex	1.15 (0.50-2.64)	.74
Disease status	1.92 (0.65-5.64)	.24
Number of HLA mismatch	2.54 (0.42-15.22)	.31
Number of infused mononuclear cells	0.59 (0.36-0.98)	.041
Number of infused $CD34^+$ cells	1.34 (0.93-1.93)	.11
GVHD prophylaxis (cyclosporine vs. tacrolimus)	0.85 (0.36-1.99)	.70
Preengraftment immune reaction	0.76 (0.33-1.76)	.52
Acute GVHD (grade II-IV)	3.48 (1.47-8.23)	.0045
Use of steroid*	1.36 (0.53-3.48)	.53
Multivariate		
Acute GVHD (grade II-IV)	3.48 (1.47-8.23)	.0045

CMV indicates cytomegalovirus; CI, confidence index; GVHD, graft-versus-host disease.
*Use of prednisolone or methyl-prednisolone >0.5 mg/kg/day.

derived anti-CMV immune cells. That contrasts with the report that recipient-derived T cells are associated with immune reaction against CMV early after transplantation following preparative regimens without TBI [27]. The issue needs to be considered in determining preparative regimens for RI-CBT. Second, transplanted cord blood stem cells are immunologically naïve. Although anti-CMV cytotoxic T-lymphocytes in transplant grafts are considered to suppress CMV proliferation early after transplantation in CMV seropositive recipients [28], passive immunity via grafts against CMV cannot be expected in CBT, and thus the risk of reactivation may be high. Third, post-transplant immune recovery is delayed in CBT. Little is known about post-CBT immune recovery with only few reports. Although the numbers of T cells, B cells, and NK cells, and their in vitro reactivity after CBT are comparable with those after BMT [29,30], post-CBT incidence of infections including CMV is high [2,7,8,31-35], and immune recovery is probably delayed compared with BMT and PBSCT. Intense reactivation itself can reportedly delay the recovery of cellular immunity [36], which might be associated with the high incidence of CMV disease in the present study. Finally, immunosuppression was intensified to control post-CBT immune reaction. In our study, GVHD prophylaxis was cyclosporine or tacrolimus alone, which was mild compared with conventional transplantation. Immune reaction occasionally occurs before and at engraftment, requiring steroid treatments [6,14,37]. In the present study, 62 patients received steroids within 100 days of CBT. Steroids might have suppressed the recovery of anti-CMV cytotoxic T-lymphocytes [38].

Some challenges remain to improve the management of CMV reactivation after RI-CBT. First, optimal methods need to be established to monitor CMV reactivation. We have introduced preemptive therapy based on the results of CMV antigenemia in our hospital. The efficacy of this method in PBSCT has been reported [17], although it might not be applicable to CBT. Of the 22 patients with CMV disease, 9 progressed from CMV antigenemia to disease despite preemptive therapy. CMV disease developed with less than 10/50,000 pp65-positive cells in 13 patients. These observations suggest that antiviral therapy might be necessary immediately after CMV antigenemia is detected in CBT. More sensitive diagnostic tests such as genetic examinations [39] are also helpful in early detection of CMV reactivation. Second, the optimal preemptive strategy that is applicable to CBT has to be established. Because the disease rate in the untreated CMV positives was 46%, preemptive administration of anti-CMV agents might be required for patients with <10/50,000 pp65-positive cells. Alternatively, universal prophylaxis of CMV might be worth investigating. Optimal dose of preemptive ganciclovir and foscarnet must be also investigated. We reduced doses of preemptive foscarnet and ganciclovir mostly because of concerns of its renal toxicity and myelotoxicity, respectively. However, the failure rate of preemptive ganciclovir or foscarnet was 18%, and it was higher than that in the studies in which those were not reduced [40,41]. Clinical impact of the dose of ganciclovir or foscarnet on preemptive therapy should be investigated in future clinical studies. Finally, identification of high-risk group for CMV reactivation is necessary. The reported risk factors in conventional HSCT include GVHD, steroid administration, CMV serostatus of recipients and donors, and age [22,28,42-44]. The high dose of transfused CD34-positive cells was an independent risk factor for CMV reactivation in our analysis (Table 3). The association between the number of CD34-positive cells and CMV infection has not been reported in previous studies on BMT and PBSCT. It remains unknown and awaits further investigations. aGVHD was an independent risk factor for CMV disease. This is comparable with the report on CMV disease after allo-SCT [25].

Most of the patients with CMV disease had CMV enterocolitis in the present study. None developed CMV pneumonia or retinitis. Although the reason for the high incidence of gastrointestinal CMV disease after RI-CBT remains unclear, the use of TBI and melphalan in the preparative regimens that have significant gastrointestinal mucous toxicity [45] and complications of gut GVHD and thrombotic microangiopathy [14] may be related. Although there are different opinions on the usefulness of antigenemia in diagnosing CMV enterocolitis [46-48], the present study demonstrated that monitoring CMV antigenemia can play a certain role in early diagnosis of CMV enterocolitis after RI-CBT. Further studies are necessary to demonstrate the pathogenesis of gastrointestinal CMV disease after RI-CBT and to develop diagnostic methods for its early detection.

Although the present study provided novel information on CMV infection after RI-CBT, some issues remain to be investigated. First, the present study was retrospective and small sized. Prospective, large-sized studies are awaited. Second, RI-CBT recipients are likely to have potential organ dysfunction because most of them are at advanced ages and have been heavy treated with chemotherapies. Such characteristics of patients may affect the treatment of CMV infection. Pharmacokinetics of antiviral agents in older patients has not been well investigated, requiring further studies. Third, recipient pretransplant CMV serostatus was reported to correlate with mortality after CBT [49]. However, anti-CMV antibodies were not examined before transplantation in this study because most patients had been heavily treated and received multiple transfusions. Pretransplant CMV serostatus needs to be investigated in future studies.

Fourth, day 100 mortality was 49% in the present study. It is higher than that in the previous study reported by the Minnesota group [5]. The exact reason of these differences remains unknown; however, it might be partly from the difference in patient's backgrounds between these studies. This high mortality rate and patient's backgrounds in the present study might have affected the results. Fifth, the management of CMV infection in the present study might have affected the incidence of CMV disease; we used the reduced dose of foscarnet or ganciclovir and anti-CMV high-titer i.v. immunoglobulin was not regularly administered. Finally, late CMV infection remains to be investigated. Because CMV antigenemia-guided pre-emptive strategy has been established [50], the prognosis of CMV infection following BMT and PBSCT improved; however, late CMV disease remains a significant issue [51]. The observation period was short in the present study, and could not provide enough information on late CMV disease.

The present study demonstrated that CMV infection is a significant complication of RI-CBT. Although RI-CBT is an attractive alternative, physicians should be alert to the fact that this transplant procedure is associated with a high risk of CMV infection.

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Invasive Fungal Infection Following Reduced-Intensity Cord Blood Transplantation for Adult Patients with Hematologic Diseases

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ABSTRACT

Invasive fungal infection (IFI) is a significant complication after allogeneic hematopoietic stem cell transplantation (HSCT); however, we have little information on its clinical features after reduced intensity cord blood transplantation (RICBT) for adults. We reviewed medical records of 128 patients who underwent RICBT at Toranomon Hospital between March 2002 and November 2005. Most of the patients received purine-analogbased preparative regimens. Graft-versus-host disease (GVHD) prophylaxis was a continuous infusion of either tacrolimus 0.03 mg/kg or cyclosporine 3 mg/kg. IFI was diagnosed according to the established EORTC/NIH-MSG criteria. IFI was diagnosed in 14 patients. Thirteen of the 14 had probable invasive pulmonary aspergillosis and the other had fungemia resulting from *Trichosporon* spp. Median onset of IFI was day 20 (range: 1-82), and no patients developed IFI after day 100. Three-year cumulative incidence of IA was 10.2%. Four of the 13 patients with invasive aspergillosis (IA) developed grade II-IV acute GVHD, and their IA was diagnosed before the onset of acute GVHD. The mortality rate of IFI was 86%. Multivariate analysis revealed that the use of prednisolone >0.2 mg/kg (relative risk 7.97, 95% confidence interval 2.24-28.4, $P = .0014$) was a significant risk factor for IA. This study suggests that IFI is an important cause of deaths after RICBT, and effective strategies are warranted to prevent IFI.

KEY WORDS

Invasive aspergillosis • Graft-versus-host disease • Corticosteroid

INTRODUCTION

Cord blood transplantation (CBT) is an attractive alternative for patients with hematologic diseases who lack a matched related or unrelated donor. The usefulness of CBT using myeloablative preparative regimens has been confirmed for pediatric patients [1,2]. Myeloablative CBT for adult patients achieves engraftment in 90% of the patients, but carries 50% risk of transplant-related mortality (TRM), mostly resulting from infection [3,4]. We and other groups have reported the feasibility of CBT using reduced-intensity regimens (RICBT) for adult patients with advanced hematologic diseases [5,6].

Because of delayed immune recovery and graft-versus-host disease (GVHD), infection is the leading cause of TRM after CBT using myeloablative preparative regimens [2-4,7]. However, studies on immune recovery following RICBT gave us hope that RICBT recipients may less frequently experience GVHD and infectious complications. Invasive fungal infection (IFI) has been 1 of the most feared infectious complications in conventional allogeneic marrow or peripheral blood stem cell (PBSC) transplantation [8,9], whereas we have little information on IFI following RICBT. We investigated its incidence and clinical features in patients who underwent RICBT for advanced hematologic diseases.

PATIENTS AND METHODS

Data Collection

We reviewed medical records of 128 recipients who underwent first reduced-intensity allogeneic hematopoietic stem cell transplantation (HSCT) using cord blood (CB) between March 2002 and November 2005 at Toranomon Hospital. Their characteristics are shown in Table 1. Of the 128 patients, 101 had high-risk diseases including acute myelogenous leukemia (AML) in relapse or the second and higher complete remission (CR; n = 42), acute lymphoid leukemia (ALL) except those in the first CR (n = 12), chronic myelogenous leukemia (CML) in blastic phase (n = 4), myelodysplasia except refractory anemia (n = 10), refractory lymphoma (n = 30), idiopathic myelofibrosis (n = 1), plasma cell leukemia in relapse (n = 1), and chronic myelomonocytic leukemia (n = 1).

Table 1. *Patients' Characteristics and Transplantation Procedures*

Variables	Number
Patients Characteristics	
Age, median (range)	56 (17-71)
Sex, male/female	80/48
Primary diseases	
AML/MDS	63
Malignant lymphoma	33
Acute lymphoblastic leukemia	17
Severe aplastic anemia	6
Chronic myelogenous leukemia	6
Chronic myelomonocytic leukemia	1
Plasmacytic leukemia	1
Idiopathic myelofibrosis	1
Risk of underlying diseases,*1 high/low	101/27
Prior autologous stem cell transplant, yes/no	9/119
Transplantation procedures	
Conditioning regimen	
Flu + Mel + TBI 2 Gy or 4 Gy/8 Gy	112/2
Flu + BU + TBI 4 Gy/8 Gy	8/1
Others	5
GVHD prophylaxis, cyclosporine/tacrolimus	64/64
Number of infused nucleated cell, median (range) $\times 10^7$/kg	2.7 (1.6-4.8)
HLA disparity (antigen), 2/1/0	108/17/3
Transplantation outcomes	
Neutrophil engraftment	99/128
Complete donor chimerism*2	90/99
Grade II-IV acute GVHD*2	45/99
Chronic GVHD*3*4	11/40
CMV antigenemia*3	48/93
CMV disease	10
Relapse*2	24/98

*1 We divided the risk of transplantation into two groups. The low-risk group was as follows: acute myelogenous or lymphoid leukemia in first and second remission, chronic myelogenous leukemia in chronic phase, and myelodysplastic syndrome refractory anemia. The other patients were defined as having high-risk diseases.

*2 Percentage was calculated based on 99 patients who achieved primary engraftment.

*3 Percentage was calculated based on the number of patients who achieved engraftment and evaluated.

*4 No patients received systemic corticosteroids for the treatment of chronic GVHD.

AML indicates acute myelogenous leukemia; MDS, myelodysplastic syndromes; ULN, upper limit of normal; GVHD, graft-versus-host disease; CMV, cytomegalovirus.

Transplantation Procedures and Supportive Cares

Transplantation procedures were shown in Table 1, and we previously reported details of the procedures [6]. GVHD prophylaxis was either tacrolimus 0.03 mg/kg or cyclosporine 3 mg/kg continuous infusion starting on day −1. Trough blood levels of these drugs were monitored 2-3 times a week and the dosage were modified to maintain the target level of 10-15 ng/mL for tacrolimus and 200-400 ng/mL for cyclosporine [10-12]. Immunosuppressants were tapered off from day 100 until day 150. If grade II-IV acute GVHD (aGVHD) developed, 1-2 mg/kg/day of methylprednisolone was added to cyclosporine or tacrolimus, and tapered from the beginning of clinical response.

The diagnosis and management of preengraftment immune reactions were reported previously [13].

Management of Infections

Patients were managed in reverse isolation laminar airflow-equipped rooms. All patients received tosufloxacin 450 mg/day from the start of conditioning until neutrophil engraftment. Fluconazole 200 mg/day or micafungin 150 mg/day, and acyclovir 600 mg/day were given from the start of conditioning until the discontinuation of GVHD prophylaxis, which were restarted when patients developed GVHD and were treated with steroids and immunosuppressants. They received prophylaxis with trimethoprim-sulfamethoxazole against *Pneumocystis jirovecii* infection from the start of conditioning until the discontinuation of immunosuppressants or disappearance of chronic GVHD (cGVHD). When patients develop neutropenic fever, tosufloxacin was changed to broad-spectrum antibiotics [14]. Intravenous administration of amphotericin B at a dose of 0.5 mg/kg/day was added when the fever persisted for more than 5 to 7 days. If the diagnosis of aspergillus infection was confirmed, the dosage of amphotericin B was increased to 1.0 mg/kg/day. We used blood tests, enzyme-linked immunosorbent assay for galactomannan antigen, (1-3)-beta-D glucan assay, and chest computed tomography for the early diagnosis of invasive aspergillosis (IA), as previously reported [15]. Because most patients had been heavily treated and received multiple transfusions prior to transplantation, anti-CMV antibodies were not examined before transplantation. All patients were monitored for cytomegalovirus pp65 antigenemia once a week. When CMV antigenemia exceeded 10/50,000, patients preemptively received foscarnet 30 mg/kg intravenously twice daily.

Diagnostic Criteria for IFI

Invasive fungal infection was diagnosed according to the established EORTC/NIH-MSG criteria [16]. Briefly, we diagnosed patients as having proved IFI when any 1 of the following examinations was positive: histopathologic or cytopathologic examinations for hyphae or yeasts in needle aspiration or biopsy specimens, fungal cultures obtained from normally sterile sites by sterile procedures, and *Cryptococcus* antigen in cerebrospinal fluid. Probable IFI was diagnosed when a patient satisfied at least 1 host factor, microbiologic criteria, and clinical criteria. Possible IFI was not included in this study. The day of diagnosis of IFI was defined as the day when the first diagnostic test was performed.

Endpoints and Statistical Analysis

The cumulative incidence of IA was evaluated using Gray's method, considering death without IA as a competing risk [17].

Potential confounding factors considered in the analysis of risk factors of IA were age, sex, disease status, previous stem cell transplantation, conditioning regimens, HLA mismatch, stem-cell dose (all nucleated cells, and CD 34-positive cells), GVHD prophylaxis, grade II-IV aGVHD, and use of prednisolone. Proportional hazard modeling was used to evaluate the influence of these factors on the incidences of IA treating the development of aGVHD and the use of prednisolone as time-dependent covariates. Factors associated with at least borderline significance ($P < .10$) in the univariate analyses were subjected to a multivariate analysis using backward stepwise proportional-hazard modeling. P-values of $<.05$ were considered statistically significant. Survival was estimated by the Kaplan-Meier method. Median follow-up of surviving patients was 628 days (range: 26-1347 days).

RESULTS

Clinical Outcomes after RICBT

Ninety-nine (77%) patients achieved primary engraftment at a median of day 20 (range: 9-53 days). Of the remaining 29 patients who failed to achieve primary engraftment, 4 patients received second RICBT, and the other 25 patients died before engraftment. Their causes of death included bacteremia (n = 22), invasive pulmonary aspergillosis (IPA) (n = 1), and progression of primary disease (n = 2). Of the 99 engrafted patients, 45 and 22 patients developed grade II and grade III-IV aGVHD, respectively. The median onset of grade II-IV aGVHD was day 28 (range, 11-92). Eleven of 40 patients (28%) who survived longer than 100 days without disease progression developed cGVHD. Estimated 3-year overall survival was 33% (95% confidence interval (95% CI), 24%-42%). Causes of deaths comprised nonrelapse mortality (n = 31) and disease progression (n = 23). Infection was the leading cause of nonrelapse mortality (n = 20). Autopsy was performed in 5 patients (3.9%) in this series of patients.

Incidence and Clinical Features of IFI

Invasive fungal infection was diagnosed in 14 patients. Their clinical features are shown in Table 2. Thirteen of the 14 patients had probable IA, and the other had fungemia from *Trichosporon* spp. Three-year cumulative incidence of probable IA was 10.2% (Figure 1). Median onset of IFI was day 20 (range: 1-82), and no patients developed IFI after day 100. IFI was diagnosed after day 30 in 1 patient. Prophylactic uses of antifungal agents included fluconazole (n = 12) and micafungin (n = 1) among the 13 patients with IA. Of the 63 patients who survived 100 days or longer, none developed IFI after day 100. Four of the 13 patients with IFI developed grade II-IV aGVHD, and their diagnosis of IFI was before the onset of aGVHD (Table 2).

Seven patients were given prednisolone >0.2 mg/kg/day for the treatment of preengraftment immune reactions, of whom 5 developed grade II-IV aGVHD. Twelve of the 14 patients with IFI died, and the mortality rate was 86%. IFI was the primary cause of deaths in 4 patients.

Risk Factors of IPA

Table 3 shows the results of univariate and multivariate analyses. Reactivation of cytomegalovirus (CMV) is a well-known risk factor of IA [18,19]. However, it was not included in the analysis of this study, because the onset of IFI was earlier than the onset of CMV antigenemia. Use of prednisolone >0.2 mg/kg (relative risk [RR], 7.97; 95% CI, 2.24-28.4; P = .0014) was a significant risk factor in multivariate analysis.

DISCUSSION

The present study demonstrated that IFI early after RICBT is a significant complication. Among IFI, the incidence of IA was high, which was consistent with the studies on reduced intensity stem cell transplantation (RIST) using other stem cell sources [9,19]. Our results contrasted with the previous reports that the incidence of infection because of non-*Candidia albicans* species was high in myeloablative allogeneic stem cell transplantation [20]. The observations may be associated with the milder gastrointestinal mucosal toxicity by conditioning regimens in RIST than in myeloablative transplantation [21] and the less fre-

Table 2. *Clinical Characteristics of Patients with Invasive Aspergillosis*

UPN	Age	Sex	Primary Disease	Disease Status at Transplant	No. of Prior Regimens	Neutrophil Engraftment Day	Grade II-IV Acute GVHD Onset Day	PSL Started	Invasive Fungal Infection (IFI)*1	Onset (Day)	Other Infectious Complication	Overall Survival (Day)	Outcomes of IFI	Causes of Death
286	57	M	AML	PIF	3				Probable IA	4		4	dead	IFI
365	69	M	AML	RL1	3				Probable IA	3		14	dead	IFI
411	56	F	ML	PD	1			11	Probable IA	19	Bacteremia	24	dead	Bacteremia complicated with IA
196	61	M	AML	PIF	2	11	22		*T. cutaneum* fungemia	1		28	dead	IFI
202	62	M	AML	RL1	4	21	21	8	Probable IA	21	Bacteremia	28	dead	Bacteremia complicated with IA
344	55	F	ML	PD	1	19			Probable IA	4	Bacteremia	30	dead	IFI
262	59	M	AML	PIF	1			10	Probable IA	20		31	dead	PD
151	52	F	MDS	RAEB	3	20		7	Probable IA	12		33	improved	GI bleeding
114	52	F	ML	PD	4	13		0	Probable IA	23		39	dead	PD
153	70	M	AML	1st CR	2	14	30		Probable IA	29	Bacteremia	46	dead	IP
197	33	M	MDS	RA	0				Probable IA	28	Bacteremia	47	dead	Bacteremia complicated with IA
160	66	M	ML	PD	2	14	29	9	Probable IA	25		75	dead	MOF
120	70	F	SAA		0	13			Probable IA	82		1308+	improved	
127	20	M	SAA		3	31	55	12	Probable IA	3		1347+	improved	

AML indicates acute myelogenous leukemia; MDS, myelodysplastic syndromes; CR, complete remission; GI, gastrointestinal; IA, invasive aspergillosis; IFI, invasive fungal infection; SAA, severe aplastic anemia; RA, refractory anemia; PD, progressive disease; PIF, primary induction failure; RARB, refractory anemia with excess of blasts; MOF, multiple organ failure.

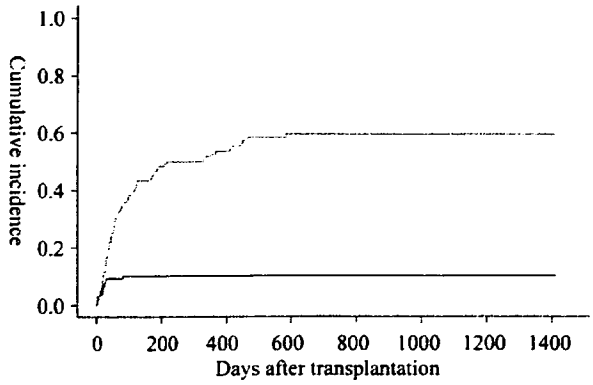

Figure 1. Cumulative incidence of invasive aspergillosis (IA) considering death without IA as a competing risk. Black line indicates incidence of death with IA, and gray line indicates incidence of death without IA.

quent and milder GVHD following CBT. Because gastrointestinal mucosal toxicity is milder in RICBT than in myeloablative transplantation, the incidence of infection from non-*Candida albicans* species as part of the gastrointestinal normal flora might be low, and hence aspergillus infection might become the majority of IFI.

The median onset of IFI was day 20 (range: 1-82) in the present study; the majority developed IFI early after RICBT. Majority of the patients who developed IFI died of causes other than fungal infection (Table 2), as reported previously by Saavedra et al. [7]. Our results were consistent with a previous report on CBT [18], and contrasted to reports on RIST using marrow or peripheral blood [19,22,23], in which IA develops late after transplantation. The low incidence of IA after day 100 would be related to the low incidence of cGVHD. The short duration from RICBT to IFI development suggests aggravation of latent infection, which would have existed before transplantation. These findings were consistent with a recent report by Martino et al. [23]. Given the possibility, several issues need to be addressed in the management of IFI following RICBT. First, selection of RICBT candidates would have to include accurate evaluation for the risk of fungal infection [24] and high-risk patients might need to be excluded from the indication of RICBT. Pretransplant CT scan of the chest and sinus would be useful in the screening of IA following RICBT, and bronchoalveolar lavage should be performed in patients with abnormal findings. Second, the importance needs to be stressed in prophylactic antifungal agents with antiaspergillus activity and attempts for early diagnosis of aspergillosis such as methods using molecular techniques [25], antigen tests [26], and imaging tests [15,22]. Third, the way of steroid use after RICBT requires further investigations. The present study showed that the administration of steroids 0.2 mg/kg and more was a strong risk factor of IA (RR, 7.97; 95% CI, 2.24-28.4; p = .0014). Our observation that the use of small-dose steroids was a risk factor of IFI after RICBT supports the previous results of severe immunosuppression early after RICBT [27]. In RICBT using our regimens, immunologic reactions such as a preengraftment immune reaction frequently develops in addition to GVHD, requiring steroid administration early after RICBT [13]. Because steroids suppress phagocyte activities and cellular immunity [28], the risk of fungal infection early after RICBT may be increased.

The incidence of late IFI was not high in the present study. Of 102 patients who survived longer

Table 3. *Univariate and Multivariate Analyses for the Incidence of IA*

	Incidence of IPA (95% CI)	P Value
Univariate analysis		
Pretransplantation factors		
Age		.30
<55	7%	
≥55	13%	
Sex		.94
Female	11%	
Male	10%	
Disease risk		.63
Standard	16%	
High	9%	
Previous ASCT		.55
No	11%	
Yes	0%	
Regimen		.72
FM-based	11%	
FB-based	10%	
HLA mismatch		.076
0 or 1 antigen	0%	
2 antigens	12%	
Cell dose		.42
ANC <2.5 × 10^7/kg	8%	
ANC ≥2.5 × 10^7/kg	12%	
Cell dose		.03
CD34 <0.8 × 10^6/kg	5%	
CD34 ≥0.8 × 10^6/kg	16%	
GVHD prophylaxis		.42
Cyclosporine	13%	
Tacrolimus	8%	
Fungal prophylaxis		.99
Fluconazole	10%	
Micafungin	10%	
Posttransplantation factors (time-dependent covariates)		
Acute GVHD		.96
Grade 0-I	1.00	
Grade II-IV	1.06 (0.12-9.40)	
Prednisolone		.001
<0.2 mg/kg/day	1.00	
≥0.2 mg/kg/day	7.97 (2.24-28.4)	
Multivariate analysis		
Prednisolone		.0014
<0.2 mg/kg/day	1.00	
≥0.2 mg/kg/day	7.97 (2.24-28.4)	

GVHD indicates graft-versus-host disease; ASCT, autologous stem cell transplantation.

than 30 days, 1 patients developed IFI after day 30. None of 63 patients who survived longer than 100 days developed IFI after day 100. Our results contrast with the previous reports on BMT and PBSC transplantation where improvement in fungal management decreased early IFI and late IFI became the majority of IFI [8,19,22,29]. In myeloablative CBT, late infection is considered a significant complication [4], whereas study results focused on fungal infection have not been published. Some hypotheses can explain the low incidence of late IFI after RICBT. First, cGVHD after RICBT is uncommon and mild. There is minimal effect of cGVHD on delay in immune recovery following RICBT. Second, steroids are not frequently administered late after RICBT for the treatment of complications such as GVHD. The incidence of cGVHD was 28% in the present study, and none of them required steroid treatments. Further studies are awaited for the clinical features of late IFI after RICBT.

The present study demonstrated clinical features of fungal infections after RICBT, leaving several issues to be investigated. First, the present study is a small-sized retrospective 1 . Unrecognized bias might affect the study results, and we obtained little information on rare fungal infections such as Fusarium and Zygomycetes. Large-sized prospective studies are awaited. Second, the diagnostic yields of IFI need to be addressed. Most of the diagnoses in our study were made based on EORTC/MSG criteria [16] using clinical, laboratory, and imaging findings. Although the clinical usefulness of the diagnostic criteria has been established, pathologic diagnosis of IFI was not confirmed in many patients and the diagnostic yields remain unclear. Underestimation of IFI incidence also remains possible, because postmortem examinations were not obtained in most patients who died without diagnosis of IFI. Because such limitations cannot be avoided in studying deep fungal infections [30], clinicians need to be aware of the limitations.

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LETTER TO THE EDITOR

Value of pretransplant screening for colonization of *Pseudomonas aeruginosa* in reduced-intensity umbilical cord blood transplantation for adult patients

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Dear Editor,

Bloodstream infection (BSI) is a serious complication after reduced-intensity cord blood transplantation (RI-CBT) [1]. Although BSI caused by gram-negative bacteria is less frequent than BSI of gram-positive organisms, it leads to considerable toxicities in RI-CBT recipients [1]. Most gram-negative organisms colonize in the alimentary tract, and mucosal damages due to preparative regimens allow their transition from colonization to BSI [2]. Surveillance cultures as pretransplant screening might be helpful for predicting the development of BSI; however, the high rates of false-positive results limit the clinical application of surveillance cultures even in patients at high-risk of chemotherapy-induced mucositis [2–5]. Some researchers reported that colonization of *P. aeruginosa*, which frequently causes fatal BSI after RI-CBT [1], might be a useful marker for predicting the development of BSI in patients who receive cytotoxic chemotherapy [2, 3]. We investigated whether the isolation of *P. aeruginosa* by pretransplant screening culture could predict BSI after RI-CBT.

Between January 2002 and March 2004, we obtained pretransplant screening cultures from 46 patients who underwent RI-CBT at Toranomon Hospital. Transplantation procedures and supportive cares were described previously [1], and patients' characteristics are shown in Table 1. Tosufloxacin 450 mg/day was given for prophylaxis against bacterial infections. All the patients provided written informed consent in accordance with the requirements of the Institutional Review Board. Definition of BSI was described previously [1]. Multidrug-resistance of *P. aeruginosa* was defined as resistance to fluoroquinolones, β-lactams, and aminoglycosides [6]. Cumulative incidence of BSI was evaluated by Gray's method, and death without BSI and relapse or progression of underlying diseases were considered as competing risks.

Pretransplant screening cultures were positive for *P. aeruginosa* in 6 of the 46 patients (13%; Table 1). Three of the six patients with positive *P. aeruginosa* cultures and 3 of the 40 patients with negative *P. aeruginosa* cultures developed BSI of *P. aeruginosa* within 30 days of RI-CBT (Table 1). The frequencies of developing BSI of *P. aeruginosa* in the two groups were significantly different (50% vs 7.5%, $p=0.022$). The positive predictive value was 50%, and the negative predictive value was 92.5%. The

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Table 1 Characteristics of the RI-CBT patients who underwent pretransplant screening culture

Variable		Patients with *P. aeruginosa* colonization (n=6)	Patients without *P. aeruginosa* colonization (n=40)
Patient backgrounds			
Age	Median (range)	54.5 (41–70)	55 (17–79)
Risk of underlying diseases[a]	High/low	5/1	28/12
Primary diseases	Acute lymphoblastic leukemia	1	3
	Acute myeloid leukemia	1	13
	Adult T cell leukemia	0	6
	Myelodysplastic syndrome	0	4
	Malignant lymphoma	2	11
	Multiple myeloma	1	1
	Aplastic anemia	1	2
Preparative regimens	Fludarabine, melphalan, TBI	6	38
	Fludarabine, busulfan, TBI	0	1
	Fludarabine, melphalan	0	1
Number of infused nuclear cells	Median (range), X10E7/kg	3.2 (2.3–4.3)	2.8 (1.7–5.2)
Number of infused CD34+ cells	Median (range), X10E5/kg	0.71 (0.4–1.23)	0.67 (0.27–3.28)
HLA matching	6/5/4	0/0/6	1/6/33
Transplantation-related mortality within 30 days		2(33%)	8(20%)
Pretransplant screening			
Cultured sites of *P. aeruginosa*	Throat/stool/urine	4/2/1	NA
Number of patients colonized with multidrug-resistant *P. aeruginosa*		2	NA
BSI due to *P. aeruginosa*			
Number of patients who developed BSI due to *P. aeruginosa* within 30 days of RI-CBT		3(50%)	3(7.5%)
	Onset day [median (range)]	4 (1–11)	18 (1–19)
Median neutrophil count at onset day (/L)		0	0
Antibiotic use for bacterial infection at onset day[b]			
	Fluoroquinolones	1	2
	β-Lactams	0	1
	Carbapenems	2	0
	Aminoglycosides	1	0
	Vancomycin	1	1
Response to antibiotics	(responded/not responded)	0/3	0/3
Mortality within 30 days in the patients with BSI due to *P. aeruginosa*		2(66%)	3(100%)

RI-CBT indicates reduced-intensity cord blood transplantation; *TBI* total body irradiation; *BSI* blood stream infection; *NA* not applicable

[a] We defined acute leukemia in complete remission, chronic myelogenous leukemia in chronic phase, malignant lymphoma in complete remission, multiple myeloma in complete remission, myelodysplastic syndrome in refractory anemia (RA) and aplastic anemia as low/high risk and the others as high risk.

[b] All the six patients received antibiotics at onset day.

BSI was fatal in five patients. Multidrug-resistant *P. aeruginosa* was cultured at pretransplant screening in two patients; one developed fatal BSI of multidrug-resistant *P. aeruginosa*, and the other died of pneumonia.

The present study suggested that pretransplant screening for *P. aeruginosa* colonization can predict its BSI and might be helpful for reducing transplant-related mortality after RI-CBT. Our results are in contrast with the previous studies that did not recommend routine surveillance cultures before cytotoxic chemotherapy and conventional allogeneic stem-cell transplantation (allo-SCT) due to its poor predictive value [2, 4]. Some hypotheses can be postulated on these observations. First, most patients in our study had received antibiotics during previous chemotherapies followed by prophylactic fluoroquinolones after RI-CBT, and they were at high risk of colonization of antibiotic-resistant organisms. Appropriate use of antibiotics is warranted to avoid colonization of such organisms.

Second, the preparative regimens including melphalan and total body irradiation, which have a significant mucosal toxicity, might have been associated with the development of BSI due to *P. aeruginosa*. As three of the six patients colonized with *P. aeruginosa* developed its BSI early after transplantation, damages to the gastrointestinal mucosa by preparative regimens might have allowed *P. aeruginosa* to enter the bloodstream. Use of preparative regimens with minimal mucosal toxicity might be beneficial to reduce the risk of BSI after RI-CBT. Alternatively, keratinocyte growth factor, which promotes the regeneration of damaged mucosa [7], is also worth investigating.

Optimal management of patients with colonization by *P. aeruginosa* has to be established. Prophylactic antimicrobial therapies directed against *P. aeruginosa* may only lead to the selection of multidrug-resistant isolates and will not improve the patients' outcomes. Rapid treatments including antibiotics against isolated *P. aeruginosa* might be important. Physicians should be alert to the clinical manifestations related to *P. aeruginosa* infection in these patients.

Infection of multidrug-resistant bacteria is a significant concern in allo-SCT. When allo-SCT recipients are colonized with methicillin-resistant *Staphylococcus aureus*, use of mupirocin calcium ointment is recommended to eliminate the bacteria [8]. Meanwhile, optimal management of multidrug-resistant *P. aeruginosa* has not been established in allo-SCT. BSI of multidrug-resistant *P. aeruginosa* causes high mortality [6], whereas the elimination of multidrug-resistant *P. aeruginosa* is difficult [9]. In the present study, BSI due to multidrug-resistant *P. aeruginosa* was fatal despite intensive antibiotic therapy. While all the patients enrolled in this study received fluoroquinolone-based prophylaxis, polymyxin might be worth investigating. It is the most consistently effective agent against *P. aeruginosa* in vitro [10]. Other options include novel antibiotic combinations, such as macrolides, tobramycin, trimethoprim, and rifampin [10]. Alternatively, RI-CBT might better be avoided in patients with colonization of multidrug-resistant *P. aeruginosa*.

We demonstrated that pretransplant screening of *P. aeruginosa* colonization could be predictive of the development of its BSI after RI-CBT. This study provided novel information to establish the risk stratification in the management of BSI after RI-CBT, although it was a small-sized, retrospective study. We plan to further investigate whether the risk stratification based on the results of pretransplant screening cultures is useful to reduce the risk of BSI after RI-CBT.

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Successful Engraftment in Reduced-Intensity Cord Blood Transplantation (CBT) as a Salvage Therapy for Graft Failure After Primary CBT in Adults

Accumulating evidence strongly supports the efficacy of umbilical cord blood transplantation (CBT) in adults (*1, 2*). This now becomes a standard alternative to bone marrow or peripheral blood stem cell transplantation for patients who lack a human leukocyte antigen (HLA)-matched donor. However, surprisingly high incidence of graft failure (GF) after CBT (7–40%) has been reported (*2–4*). The second CBT could be a therapeutic strategy to rescue patients with GF, but very few cases of successful engraftment by the second CBT for patients with GF after primary CBT have been reported (*4–6*).

In the past few years, we performed the second CBT in four cases with primary GF after CBT and all cases successfully achieved engraftment as summarized in Table 1. In these salvage CBTs, we paid attention to following three points. First, we tried to make a confirmation of GF and decision to perform the salvage CBT as quickly as possible. The confirmation of GF was made by no donor chimerism in bone marrow cells on day 28 or by no sign of hematopoietic recovery until day 35 (week 5) after primary CBT. Finally, the salvage CBT was performed before day 42 (week 6). The earlier application of salvage CBT while patients still have better performance status without infection or organ toxicities may improve the engraftment and survival.

Second, considering toxicities of conditioning regimen used for primary CBT, reduced-intensity CBT was chosen for the second transplant to avoid regimen-related toxicity and mortality. Because strong immunosuppression has a clear advantage over engraftment, we used fludarabine-based preparative regimen. Subsequent conditioning therapy including fludarabine within a short duration after primary transplant and strong graft-versus-host disease (GVHD) prophylaxis could cause a high risk of infection, particularly cytomegalovirus (CMV) in CBT. However, only subclinical CMV infection occurred, which was well controllable with preemptive administration of ganciclovir. Acute GVHD was also mild.

Third, to intensify the immunosuppression in combination with a key drug tacrolimus, we utilized mycophenolate mofetil (MMF) instead of methotrexate (MTX) which was used in the first CBT in cases 1, 2 and 3 for the following two reasons.

1. MMF has been reported to cause lower incidence of mucositis compared with MTX (*7*).
2. Although mechanism has not been elucidated, several reports have suggested that a GVHD prophylaxis regimen containing MMF after allogeneic transplantation is associated with faster engraftment (*7–10*).

Our retrospective observation also shows the promotional effect of MMF in hematopoietic engraftment (data not shown), but further studies are necessary to decide the optimal dose of MMF for stem cell transplantation.

TABLE 1. Patient characteristics

	Case 1		Case 2		Case 3		Case 4	
Age/sex	55/female		53/male		45/female		23/female	
Disease status	ALL, 2nd CR		APL, 2nd CR		DLBL, 2nd CR		SAA-post CBT secondary GF	
Transplantation	1st	2nd	1st	2nd	1st	2nd	2nd	3rd
Conditioning regimen	TBI-CY	Flu-BU	TBI/CY	Flu-BU	TBI-CY	Flu-BU	TBI-Flu-Mel	TBI-Flu-Mel
HLA matching	5/6	5/6	4/6	4/6	4/6	5/6	4/6	4/6
Total cell dose ($\times 10^7$/kg)	2.81	2.44	2.07	2.01	4.01	2.28	2.41	4.1
CD34$^+$ cell dose ($\times 10^5$/kg)	3.7	0.43	0.77	0.52	0.63	1.16	0.63	1.64
GVHD prophylaxis	CyA+sMTX	FK506+MMF	CyA+sMTX	FK506+MMF	FK506+sMTX	FK506+MMF	FK506	FK506+MMF
Day of second transplant	Day 37		Day 39		Day 39		Day 42	
Days to neutrophils $>0.5\times 10^9$/L	Day 42		Day 32		Day 31		Day 19	
Days to platelets $>20\times 10^9$/L	Day 129		Not reached		Not reached		Day 166	

ALL, acute lymphoblastic leukemia; APL, acute promyelocytic leukemia; DLBL, diffuse large B-cell lymphoma; SAA, severe aplastic anemia; CR, complete remission; CBT, cord blood transplantation; GF, graft failure; TBI, total body irradiation; Flu, fludarabine; BU, busulfan; Mel, melphalan; GVHD, graft-versus-host disease; CyA, cyclosporine A; sMTX, short-term methotrexate; MMF, mycophenolate mofetil; FK506, tacrolimus.

We all have to recognize the fact that GF can possibly occur in approximately one third of adult CBT, particularly in the case of low transplant cell numbers. It would be important to make sure of a cord blood unit for salvage transplant as early as possible, and not to lose a chance to make a decision of the salvage CBT to avoid life-threatening complications. Further clinical studies are necessary to establish reduced-intensity CBT as a salvage therapy for primary GF.

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Mechanism of Decrease of Oral Bioavailability of Cyclosporin A During Immunotherapy upon Coadministration of Amphotericin B

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ABSTRACT: The trough level of blood concentration of cyclosporin A (CyA) in a patient receiving immunotherapy was observed to decrease following coadministration of amphotericin B (AMB). This clinical observation was confirmed experimentally in Wistar rats intravenously given AMB (1.5 or 3.0 mg/kg) or saline (control) for 4 days, followed by CyA (10 mg/kg). The blood concentration of CyA after i.v. or p.o. administration in both AMB groups was significantly decreased compared with the control. The oral bioavailability of CyA after 1.5 or 3.0 mg/kg AMB treatment was decreased to 67% or 46%, respectively, of that of the control group. AMB treatment increased the expression levels of mdr1a and mdr1b mRNAs in the duodenum to about three times the control, and expression of CYP3A2 mRNA in the liver was increased to about twice the control. The P-gp and CYP3A2 proteins were increased significantly. These findings suggest that the oral bioavailability of CyA is reduced as a result of both increased efflux transport via P-glycoprotein in the duodenum and an increased first-pass effect of CYP3A2-mediated hepatic metabolic activity, induced by AMB. It is suggested that careful monitoring of CyA levels is necessary in the event of AMB administration to patients receiving immunotherapy with CyA. Copyright © 2008 John Wiley & Sons, Ltd.

Key words: cyclosporin A; amphotericin B; P-glycoprotein; CYP3A; drug interaction; oral bioavailability

Introduction

There have been many reports of drug interactions involving cyclosporin A (CyA), and clinically, a patient was encountered who was receiving immunotherapy with CyA in whom the blood concentration of CyA was decreased upon coadministration of amphotericin B (AMB) (Figure 1).

It is well known that CyA is a substrate of both the efflux transporter P-glycoprotein (P-gp) and the metabolic enzyme cytochrome P450 (CYP3A) [1–3]. P-gp and/or CYP3A limit the oral bioavailability of digoxin [4], rifampin [4], vinblastine [5], dextromethorphan [6] and CyA [7,8]. It has already been shown that the blood concentration

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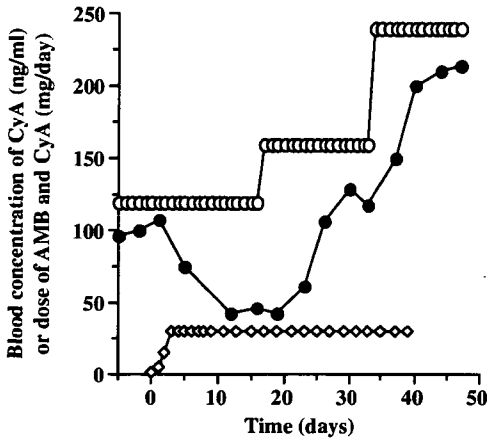

Figure 1. Change of blood concentration–time courses of CyA in a patient (23-year-old man, 56 kg) receiving immunotherapy with CyA, upon coadministration of AMB. He received daily oral administration of CyA, and then was intravenously infused over 6 h with AMB (daily for the first 10 days, every other day for the next 15 days). ● CyA blood concentration; ○ CyA dose; ◇, AMB dose

of CyA is reduced by pretreatment with dexamethasone [9], cyclophosphamide [10] or levothyroxine [11] in rats, owing to induction of P-gp and CYP3A2 in the liver and intestine. In those reports, it was demonstrated that the oral bioavailability of CyA is primarily controlled by the level of CYP3A in the upper small intestine under physiological conditions, whereas after treatment with inducers, P-gp in the upper intestine also plays a significant role as an absorption barrier to CyA. Thus, it was speculated that the reason for the decrease of blood concentration of CyA in the patient mentioned above might have been induction of P-gp and CYP. However, no report is currently available on the drug interaction between CyA and AMB.

Therefore, this study examined the mechanism of the decrease of blood concentration of CyA by using rats treated with AMB.

Materials and Methods

Chemicals

Fungizone® injection (amphotericin B, AMB) and Sandimmun® injection (cyclosporin A, CyA) were purchased from Bristol-Myers Squibb Co. Ltd (Tokyo, Japan) and Novartis Pharma Co. Ltd (Tokyo, Japan), respectively. Oligonucleotide primers were custom-synthesized by Amersham Pharmacia Biotech (UK). Other reagents were purchased from Sigma Co. (MO, USA).

Animal experiments

All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Kanazawa.

A 150 µl aliquot of AMB (1.5 or 3.0 mg/kg/day) was intravenously administered to male 8-week-old Wistar rats (Japan slc Co., Hamamatsu, Japan) via a tail vein daily for 4 days. The vehicle control rats received distilled water alone for 4 days. A 100 µl aliquot of CyA (10 mg/kg) was injected via the femoral vein at 24 h after the last treatment with AMB. Alternatively, a 500 µl aliquot of CyA (10 mg/kg) was administered orally at 24 h after the last treatment with AMB. Blood samples (200 µl each) were collected at designated time intervals from the jugular vein of untreated rats and AMB-treated rats under light ether anaesthesia.

Measurement of blood concentration of CyA

The blood concentration of CyA was measured with a TDx analyser using a commercial kit according to the manufacturer's instructions (Dainabot Co. Ltd, Tokyo, Japan). The TDx assay is a fluorescence polarization immunoassay (FPIA) reagent system for the measurement of CyA [12]. The measurement range of blood concentration was 25–1500 ng/ml. The cross-reactivities with the metabolites of CyA were 19.4% for M1 and less than 5% for other metabolites.

Determination of laboratory data

Blood samples were collected from the jugular vein under light ether anesthesia at 24 h after the last treatment with AMB, and the plasma was separated by centrifugation and stored at −80°C. The measurements of laboratory data were entrusted to SRL Co. Ltd (Tokyo, Japan).

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Reverse transcriptase-polymerase chain reaction (RT-PCR) assay

Total RNA was isolated from the liver and intestine using an Isogen kit (Wako, Osaka). Synthesis of cDNA from the isolated total RNA was carried out using RNase H-reverse transcriptase (Gibco BRL, Rockville, MD). Reverse transcription (RT) reactions were carried out in 40 mM KCl, 50 mM Tris-HCl (pH 8.3), 6 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM each of dATP, dCTP, dGTP and dTTP, 10 units of RNase inhibitor (Promega, Madison, WI), 100 pmol of random hexamer, total RNA and 200 units of the Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Berlin, Germany) in a final volume of 50 µl at 37°C for 60 min. A polymerase chain reaction (PCR) was carried out in a final volume of 20 µl, containing 1 µl of RT reaction mixture, 50 mM KCl, 20 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 10 µM each of the mixed oligonucleotide primers, and 1 unit of Taq DNA polymerase (Gibco-BRL). Reported primers were used for rat mdr1a (511 bp) [13], for rat mdr1b (451 bp) [13], for rat CYP3A2 (252 bp) [14] and for rat ß-actin (456 bp) [15]. Each cycle consisted of 30 s at 94°C, 60 s at 62°C and 75 s at 72°C for mdr1a, mdr1b and CYP3A2, and 30 s at 94°C, 60 s at 58°C, and 75 s at 72°C for ß-actin. The PCR reaction was run for 26 cycles for liver, for 34 cycles for intestine and for 22 cycles for ß-actin.

Preparation of microsomes and plasma membrane fraction

For preparation of microsomes, the liver was homogenized in three volumes of 100 mM Tris-HCl buffer (100 mM KCl, 1 mM EDTA, pH 7.4). Microsomes were prepared as reported previously [16] and stored at −80°C until use. The intestine was quickly removed and washed with buffer containing 2 mM HEPES, 0.9% NaCl and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The mucosa was scraped off with a slide glass on ice and homogenized in a buffer containing 300 mM mannitol, 5 mM EDTA, 5 mM HEPES and 1 mM PMSF (pH 7.1). The homogenate was centrifuged at $10\,000 \times g$ for 20 min, and the supernatant was centrifuged at $105\,000 \times g$ for 60 min at 4°C. The pellet was added to the buffer containing 500 mM KCl, 1 mM EDTA, 2 mM DTT and 50 mM KPB (pH 7.4) and again centrifuged at $105\,000 \times g$ for 60 min at 4°C. The resulting pellet was added to the buffer containing 1 mM EDTA, 2 mM DTT and 50 mM KPB (pH 7.4), and stored at −80°C until use.

For preparation of the plasma membrane, the liver was homogenized in 10 mM Tris-HCl buffer (pH 7.5) containing 2 mM $CaCl_2$ at 4°C. The homogenate was centrifuged at $3000 \times g$ for 10 min, and the supernatant was then centrifuged at $15\,000 \times g$ for 30 min. The pellet was washed, resuspended in 50 mM Tris-HCl buffer (pH 7.2), and twice centrifuged at $10\,000 \times g$ for 5 min, then stored at −80°C until use. The intestine was quickly removed and washed with ice-cold isotonic saline containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The mucosa was scraped off with a slide glass on ice and homogenized in a buffer containing 250 mM sucrose, 50 mM Tris-HCl (pH 7.4) and 1 mM PMSF. The homogenate was centrifuged at $3000 \times g$ for 10 min, and the supernatant was again centrifuged at $15000 \times g$ for 30 min. The resulting pellet was resuspended in 0.5 ml of a buffer containing 50 mM mannitol, 50 mM Tris-HCl (pH 7.4) and 1 mM PMSF, and stored at −80°C until use. Protein contents were measured according to the method of Lowry *et al.* [17].

SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting with peroxidase/antiperoxidase staining of the plasma membrane for P-gp and of the microsomes for CYP3A were carried out essentially as described by Laemmli [18] and Guengerich *et al.* [19]. The amounts of sample protein of liver and intestine were 10 and 25 µg for CYP3A or 40 and 80 µg for P-gp, respectively. The sample protein was electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto PVDF membrane filters (Millipore Co., Billerica, MA). After having been blocked with 5% skim milk, the filters were incubated overnight at 4°C with primary antibody, mouse anti-P-gp C219 (Abcam, Cambridge, UK) and rabbit anti-rat CYP3A2 antibody (Daiichi Pure Chemicals Co. Ltd, Tokyo, Japan), and for 1 h with secondary antibody, anti-mouse IgG HRP-linked antibody

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