interleukin (IL) 2, IL-6, and tumor necrosis factor α, reduced levels of IFN-γ and IL-10, and complete absence of IL-4 and IL-5 (30, 31). Pre-engraftment fever is possibly attributable to a cytokine storm induced by massive proliferation of cells with a unique cytokine profile. Another possibility is homeostasis-driven proliferation of naive T cells in highly immunosuppressed individuals, as demonstrated in murine models (32, 33). This reaction is reportedly associated with cytotoxic cytokines (32, 33). Fever as a transient response to contamination with maternal blood or cells during CB collection cannot be excluded (34). Reactivation of human herpesvirus 6 might be associated with this complication (35). If pre-engraftment fever exerts some antitumor effects, it is reasonable that patients with advanced and chemorefractory hematological diseases achieved long-term remission after RI-UCBT in the present study.

Infection is a common and significant problem in myeloablative UCBT (8, 9, 24), but little is known in RI-UCBT. The present study demonstrated that infection is also problematic in RI-UCBT. Twelve patients developed infection in this study, 9 of whom had been on corticosteroid therapy. Eight of 11 patients with CMV antigenemia had received corticosteroids. Delayed immunological reconstitution with or without GVHD, pre-engraftment fever, and corticosteroids may be risk factors for infection. Appropriate management of GVHD and pre-engraftment fever warrants additional investigation.

One-year OS was 35% in the present study, showing that some patients with advanced hematological malignancies can achieve durable remission after RI-UCBT. Contrary to our prediction, primary diseases recurred only in 3 patients. The candidates for RI-UCBT have extremely poor prognosis with conventional salvage chemotherapy. These findings suggest that RI-UCBT exerts strong antitumor activity and is promising for patients with refractory hematological malignancies without an HLA-identical sibling or an unrelated donor. In contrast, it is premature to apply RI-UCBT to low-risk diseases.

In conclusion, our study demonstrated the feasibility of RI-UCBT for adult patients with advanced hematological diseases, although the limitations included the small sample size and short follow-up. If CB is feasible for adults as an alternative stem cell source, RI-UCBT may become the choice of treatment for patients with advanced hematological diseases that are incurable with conventional treatments. RI-UCBT is particularly appealing for patients who require urgent treatments. Although RI-UCBT is currently associated with a high TRM, this study provided a rationale for continuing our clinical trials. Additional investigations need to focus on minimizing adverse effects including RRT, GVHD, and pre-engraftment immune reactions, whereas preserving graft-*versus*-leukemia effects.

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Early Central Nervous System Complications after Reduced-Intensity Stem Cell Transplantation

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ABSTRACT

To investigate clinical characteristics of early central nervous system (CNS) complications after reduced-intensity stem cell transplantation (RIST), we reviewed the medical records of 232 patients who had undergone RIST for hematologic diseases at our institutions between September 1999 and June 2003. All patients had received purine analog–based preparative regimens. Stem cell sources comprised granulocyte colony-stimulating factor–mobilized blood from HLA-identical or 1 locus–mismatched related donors (n = 151), unrelated bone marrow (n = 44), or unrelated cord blood (n = 37). Graft-versus-host disease prophylaxis incorporated cyclosporine with or without methotrexate. Diagnosis of CNS complications was based on clinical, radiologic, and microbiological findings. CNS complications occurred in 18 patients (7.8%), with a median onset of 22 days, and were infectious (n = 1), metabolic (n = 15), or cerebrovascular (n = 2). Symptoms included seizures (n = 7), visual disturbance (n = 2), headache (n = 8), nausea (n = 8), vomiting (n = 6), impaired consciousness (n = 16), and hemiparesis (n = 3). Complications improved promptly in 10 patients, and 8 patients died without improvement within 30 days. Multivariate analysis with logistic regression identified umbilical cord blood transplantation as a significant risk factor for early CNS complications (odds ratio, 14.5; 95% confidence interval, 3.7-56.9; $P < .0001$). CNS complications are a significant problem after RIST, particularly with umbilical cord blood. Limbic encephalopathy is an unrecognized subtype of neurotoxicity after umbilical cord blood transplantation.

KEY WORDS

Allogeneic hematopoietic stem cell transplantation • Graft-versus-host disease • Umbilical cord • Cyclosporine neurotoxicity • Limbic encephalopathy

INTRODUCTION

Research in the area of neurologic complications is limited with regard to allogeneic hematopoietic stem cell transplantation (allo-HSCT). Most studies have been either retrospective or reliant on autopsy records [1–6]. Prospective evaluation of this complication has been rare [7,8]. The incidence of neurologic complications has varied from 37% to 91%, and such complications have been the cause of death in 6% to 26% of patients [1,3,8]. These findings indicate that neurologic complications represent a significant problem in conventional myeloablative allo-HSCT.

Neurologic complications occur at 3 stages of allo-HSCT: (1) after the use of conditioning agents for marrow ablation, (2) during posttransplantation pan-

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cytopenia, or (3) after immunosuppressive therapies and graft-versus-host disease (GVHD) [1–3,9]. These complications are usually categorized into 4 groups: (1) infectious, (2) cerebrovascular, (3) metabolic, or (4) immune-mediated disorders. Among these 4 types of neurotoxicity, cerebrovascular disorders and central nervous system (CNS) infection before engraftment have represented significant problems in conventional allo-HSCT [1,4,8]. Whether GVHD can affect the CNS remains controversial [10], and neurotoxicity has thus been regarded as an early complication after allo-HSCT.

A new transplantation strategy using a nonmyeloablative preparative regimen—reduced-intensity stem cell transplantation (RIST)—was developed to decrease regimen-related toxicity while preserving adequate antitumor effects [11,12]. Different pioneering conditioning regimens for RIST have been investigated, such as those including purine analogs [11–13] and total body irradiation (TBI) combined with potent immunosuppressants [14]. Although early reports on RIST emphasized safety advantages [11,15], recent studies have revealed considerable toxicities associated with this type of transplantation [16,17]. Little information is available on CNS complications after RIST. We investigated early CNS complications after RIST with regard to incidence, characteristics, and risk factors.

PATIENTS AND METHODS

Patients

Medical records of all patients who underwent RIST for treatment of hematologic diseases at the National Cancer Center Hospital or Toranomon Hospital between September 1999 and June 2003 were reviewed. Subjects comprised 232 patients (143 men and 89 women) with a median age of 54 years (range, 15-73 years). Primary diseases consisted of acute myeloid leukemia (n = 63), chronic myelogenous leukemia (n = 15), acute lymphoblastic leukemia (n = 8), malignant lymphoma (n = 67), myelodysplastic syndrome (n = 42), adult T-cell leukemia/lymphoma (n = 17), multiple myeloma (n = 10), aplastic anemia (n = 8), and others (n = 2). Hematologic malignancies were refractory to cytotoxic chemotherapy in 142 patients and were in remission or sensitive to treatment in 81 patients. Underlying diseases were not malignant in the remaining 9 patients.

Transplantation Procedures

All patients had received purine analog–based preparative regimens comprising fludarabine/cyclophosphamide (n = 12) [18], fludarabine/busulfan (n = 139) [19], fludarabine/melphalan (n = 55) [20], cladribine/busulfan (n = 25) [13], and others (n = 1). Rabbit antithymocyte globulin and TBI (4-8 Gy) were added to preparative regimens in 50 and 65 patients, respectively.

Stem cell sources were HLA-identical or 1 locus–mismatched granulocyte colony-stimulating factor–mobilized peripheral blood (n = 151), unrelated bone marrow (n = 44), or unrelated umbilical cord blood (n = 37). GVHD prophylaxis was cyclosporine alone (3 mg/kg) in RIST from an HLA-identical related donor and reduced-intensity umbilical cord blood transplantation (RI-UCBT). Patients who received transplants from a 1 locus–mismatched related donor or a matched unrelated donor received cyclosporine and short-term methotrexate. Grade II to IV acute GVHD was treated with methylprednisolone 2 mg/kg/d in addition to cyclosporine.

Diagnostic Criteria for Early CNS Complications

Early CNS complications were defined as CNS toxicity occurring within 100 days of transplantation. Diagnosis of CNS complications was made by clinical, radiologic, or microbiological findings (or a combination of these). CNS complications were categorized into 4 groups: (1) infectious, (2) cerebrovascular, (3) metabolic, and 4) immune-mediated disorders. CNS complications that occurred after relapse or progression of underlying diseases were excluded from analysis. Diagnosis of cyclosporine encephalopathy was based on the typical radiologic findings, ie, symmetrical white matter lesions mainly localized in the occipital lobe. In the case of limbic encephalopathy, the diagnosis was based on selective involvement of the medial temporal lobe on magnetic resonance imaging (MRI). Diagnosis of cerebrovascular diseases was confirmed by neuroradiologic or postmortem studies (or both). Abnormalities on imaging were defined as areas of low white-matter attenuation on computed tomographic (CT) scans and as areas of T1-weighted hypointensity and T2-weighted hyperintensity on MRI.

End Points and Statistical Analysis

The primary end point of this study was incidence of early CNS complications after RIST. A secondary objective was to investigate characteristics and risk factors for such complications. The median follow-up of surviving patients was 17.5 months (range, 8.5-52.7 months).

Univariate analysis with χ^2 and Mann-Whitney tests was performed to identify risk factors for CNS toxicity. Variables included age, sex, primary disease, disease status (refractory or sensitive to cytotoxic chemotherapy), and type of transplantation. We added multiple logistic regression analysis to assess the fractionated contribution of the above-mentioned potentially predictive factors. Variables that had a P value of

<.25 on univariate analysis were entered into the mixed-effects model. Those that contributed <10% to the overall ability of the model to influence serum levels of fluconazole were sequentially eliminated. The level of significance was set at $P < .05$.

RESULTS

Incidences and Types of CNS Complications after RIST

A total of 18 patients (7.8%) developed early CNS complications. Subtypes comprised infectious (invasive aspergillosis; n = 1), metabolic (n = 15; cyclosporine neurotoxicity, n = 4; limbic encephalopathy, n = 4; hemophagocytic syndrome, n = 1; leukoencephalopathy, n = 1; idiopathic, n = 5), and cerebrovascular (subdural hematoma, n = 1; subarachnoid hemorrhage, n = 1) complications. No patient was diagnosed with immune-mediated CNS toxicity.

Clinical and Laboratory Features at Onset of CNS Complications

Backgrounds of the patients who developed CNS complications are shown in Table 1. Except for a patient with aplastic anemia, the remaining 17 patients had refractory hematologic diseases.

Clinical and laboratory findings at the onset of CNS complications are shown in Table 2. The median onset was 22 days (range, 1-74 days). Seizures developed in 7 patients (generalized, n = 6; focal, n = 1). Other symptoms included headache (n = 8), nausea (n = 8), vomiting (n = 6), impaired consciousness (n = 16), and hemiparesis (n = 3). Two of 11 evaluable patients developed visual disturbance (blurred vision). Cyclosporine blood levels were higher than target levels (250-350 ng/mL) in 4 patients. Nine patients displayed fever at the onset of CNS complications, and 2 patients were receiving steroid therapy for acute GVHD. Concomitant conditions in the 15 patients with metabolic encephalopathy included systolic hypertension (>170 mm Hg) in 6 patients, diastolic hypertension (>100 mm Hg) in 6, hyponatremia in 8, hypomagnesemia in 6, and hypocholesterolemia in 4. Cerebrospinal fluid obtained from 5 patients showed normal levels of protein and cell counts. No pathogens such as bacteria, fungi, or viruses were cultured from cerebrospinal fluid.

Imaging Studies

Seventeen patients underwent cranial imaging studies: CT only in 6, MRI only in 4, and both CT and MRI in 7. Results are shown in Table 2. Of the 14 patients with metabolic encephalopathy who underwent imaging studies, 7 displayed some abnormal findings. Lesions were located bilaterally in the occipital lobes (n = 3), temporal lobes (n = 3), or periventricular white matter (n = 1). Three patients who had received UCBTs were diagnosed with limbic encephalopathy on the basis of imaging studies (Figure 1).

Treatment and Outcomes

Cyclosporine was continued (n = 4) or withheld (n = 14) for 1 to 14 days. Two patients received antihypertensive agents. Corticosteroids were used in 16 patients. In most patients, subsequent treatment with cyclosporine was well tolerated without recurrence of neurotoxicity.

Eight patients died within 30 days of developing CNS complications. Causes of death included disease progression (n = 1), subarachnoid hemorrhage (n = 1), GVHD (n = 3), and infection (n = 3). CNS complication was a primary cause of death in 2 cases (invasive aspergillosis, n = 1; subarachnoid hemorrhage, n = 1).

Risk Factors

In univariate analysis, the development of CNS complications was associated with the use of umbilical cord blood ($P < .0001$) and the status of underlying disease (chemorefractory hematologic diseases versus others; $P = .032$). Multivariate analysis showed that the use of umbilical cord blood was significantly correlated with CNS complications after RIST (odds ratio, 14.5; 95% confidence interval, 3.7-56.9; $P < .0001$).

DISCUSSION

In this study, CNS complications occurred in 7.8% of RIST recipients, and mortality with 30 days of its development reached 44%. These findings indicate that early CNS complications are a common and important problem in both RIST and conventional allo-HSCT [1,3,4,8]. However, significant differences existed in clinical characteristics of CNS complications between RIST and conventional myeloablative allo-HSCT.

The incidence of CNS complications was lower in RIST than in conventional allo-HSCT, in which 11% to 44% of patients develop such complications [2,6,7]. In conventional transplantation, the most common causes of CNS complications are cerebrovascular disease and infection after conventional transplantation [1,4,8], and these are mostly attributable to regimen-related toxicity [21,22] or profound myelosuppression before engraftment [1,3,4]. However, in RIST, regimen-related toxicities are minimal, and myelosuppression is short. Acute GVHD, as the most important complication in RIST [16], rarely affects the CNS [23]. RIST has, at the very least, improved the safety of allo-HSCT by decreasing the incidence of CNS complications.

Table 1. *Backgrounds of Patients Who Developed CNS Complications after RIST*

Patient No.	Type of CNS Complication	Age (y)	Sex	Primary Disease	History of CNS Involvement	No. of Chemotherapy Regimens before Transplantation	Preparative Regimen	GVHD Prophylaxis	Stem Cell Source
1	Cerebrovascular	57	M	ALL	Yes	1	Flu/BU/ATG	Cyclosporine	HLA-identical sibling
2	Cerebrovascular	32	F	Malignant lymphoma	No	1	Flu/Mel/TBI 4 Gy	Cyclosporine	Umbilical cord blood
3	Infectious	40	M	MDS	No	2	Flu/Mel/TBI 4 Gy	Cyclosporine	Umbilical cord blood
4	Metabolic	21	M	Aplastic anemia	No	1	Flu/BU/ATG	Cyclosporine	HLA-identical sibling
5	Metabolic	67	M	Malignant lymphoma	No	1	Flu/Mel/TBI 4 Gy	Cyclosporine	Umbilical cord blood
6	Metabolic	67	M	MDS	No	1	Flu/BU/TBI 4 Gy/ATG	Cyclosporine/Methotrexate	Matched unrelated donor
7	Metabolic	51	M	AML	No	2	Flu/BU/TBI 4 Gy	Cyclosporine	Umbilical cord blood
8	Metabolic	52	M	MDS	No	2	Flu/ATG	Cyclosporine	Mismatched related donor
9	Metabolic	49	M	ALL	No	1	Flu/BU	Cyclosporine	HLA-identical sibling
10	Metabolic	48	F	AML	Yes	3	Flu/BU/ATG	Cyclosporine/Methotrexate	Mismatched related donor
11	Metabolic	57	F	AML	No	1	Flu/Mel/TBI 4 Gy	Cyclosporine	Umbilical cord blood
12	Metabolic	66	M	Malignant lymphoma	No	2	Flu/Mel/TBI 4 Gy	Cyclosporine	Umbilical cord blood
13	Metabolic	63	M	MDS	No	1	Flu/Mel/TBI 4 Gy	Cyclosporine	Umbilical cord blood
14	Metabolic	54	M	AML	No	1	Flu/Mel/TBI 4 Gy	Cyclosporine	Umbilical cord blood
15	Metabolic	55	M	Malignant lymphoma	No	1	Flu/Mel/TBI 4 Gy	Cyclosporine	Umbilical cord blood
16	Metabolic	62	F	ATL	No	1	Flu/Mel/TBI 4 Gy	Cyclosporine	Umbilical cord blood
17	Metabolic	46	M	ATL	No	1	Flu/Mel/TBI 4 Gy	Cyclosporine	Umbilical cord blood
18	Metabolic	54	F	ATL	No	1	Flu/Mel/TBI 4 Gy	Cyclosporine	Umbilical cord blood

AML indicates acute myeloblastic leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia; ATL, adult T-cell leukemia/lymphoma; Flu, fludarabine; BU, busulfan; ATG, antithymocyte globulin; TBI, total body irradiation; CNS, central nervous system.

Table 2. *Clinical, Laboratory, and Radiologic Characteristics at the Onset of CNS Complications*

				Clinical Findings							Laboratory Findings						Radiologic Examination			
Patient No.	Type of CNS Complication	Cause	Onset (day)	Impaired Consciousness	Seizures	Visual Disturbance	Fever (>38.0°C)	Blood Pressure (Systolic; mm Hg)	Blood Pressure (Diastolic; mm Hg)	Cyclosporine (ng/mL)*	Creatinine (mg/dL)	Hemoglobin (g/dL)	Na (mEq)	K (mEq)	Mg (mEq)	T-Chol (mg/dL)	CT	T2-Weighted MRI	Electroencephalogram	Outcomes
1	Cerebrovascular		16	No	No	No	No	170	87	386	0.6	9.0	139	4.3	1.5	NA	NA	Subdural hematoma	NA	Improved
2	Cerebrovascular		40	Yes	Yes	Yes	Yes	152	98	NA	1.6	7	137	3.5	1.3	217	Brain edema, subarachnoid hemorrhage	NA	NA	Dead
3	Infection		68	Yes	No	No	No	108	64	NA	1.2	7.5	136	3.4	0.6	140	Mass in the parietal lobe	NA	NA	Dead
4	Metabolic encephalopathy	Cyclosporine	8	No	No	No	Yes	142	74	316	0.8	6.5	140	4.0	1.5	NA	NA	Bilateral parietal and occipital lobes	NA	Improved
5	Metabolic encephalopathy	Limbic encephalopathy	22	Yes	Yes	No	No	170	108	219	0.3	8.2	124	3.5	1.2	143	NA	Bilateral temporal lobes	NA	Improved
6	Metabolic encephalopathy	Cyclosporine	22	Yes	Yes	Not evaluable	Yes	182	100	266	1.2	7.5	141	2.5	1.8	NA	Low-density area in the bilateral occipital lobes	NA	NA	Improved
7	Metabolic encephalopathy	Cyclosporine	22	Yes	Yes	Not evaluable	No	120	80	348	1.5	6.5	139	4.8	1.3	107	Normal	Bilateral occipital lobes	NA	Improved
8	Metabolic encephalopathy	Cyclosporine	7	Yes	Yes	Not evaluable	Yes	170	78	342	0.8	4.1	138	3.6	1.3	145	Normal	NA	NA	Improved
9	Metabolic encephalopathy	Hemophagocytic syndrome	46	Yes	No	Yes	No	130	64	NA	0.7	9.7	139	4.1	NA	110	NA	Normal	NA	Dead
10	Metabolic encephalopathy	Leukoencephalopathy	12	Yes	No	No	Yes	110	56	NA	1.1	6.5	134	4.0	NA	NA	Normal	Bilateral frontal and parietal lobes (periventricular area)	NA	Dead
11	Metabolic encephalopathy		20	Yes	No	No	No	154	100	584	0.8	8.2	130	2.9	NA	157	Normal	NA	NA	Dead
12	Metabolic encephalopathy		13	Yes	No	Not evaluable	Yes	190	120	511	1.5	8.1	130	4.2	NA	162	Normal	Normal	Normal	Improved
13	Metabolic encephalopathy	Limbic encephalopathy	24	Yes	No	No	No	158	85	68	0.8	9.7	131	4.3	NA	NA	Normal	Bilateral temporal lobes	Diffuse slow waves	Improved
14	Metabolic encephalopathy		22	Yes	Yes	Not evaluable	Yes	168	86	416	0.8	7.8	134	3.9	1.3	107	Normal	Normal	Diffuse slow waves	Dead
15	Metabolic encephalopathy		41	Yes	No	No	Yes	180	120	52	NA	NA	NA	NA	NA	NA	Normal	NA	Diffuse slow waves	Dead
16	Metabolic encephalopathy	Limbic encephalopathy	26	Yes	Yes	Not evaluable	Yes	174	98	37	2.4	7.4	127	3.4	1.4	130	Normal	Bilateral temporal lobes	Spike wave in frontal lobes	Dead
17	Metabolic encephalopathy		18	Yes	No	Not evaluable	No	150	100	156	0.4	9.7	113	3.7	NA	NA	Normal	Normal	Diffuse slow waves	Improved
18	Metabolic encephalopathy		74	Yes	No	No	No	130	80	NA	2.2	12.1	119	5.4	NA	NA	NA	NA	NA	Improved

NA indicates not applicable; T-chol, total cholesterol.
*Continuous infusion of cyclosporin was given at target levels of 250-350 ng/mL.

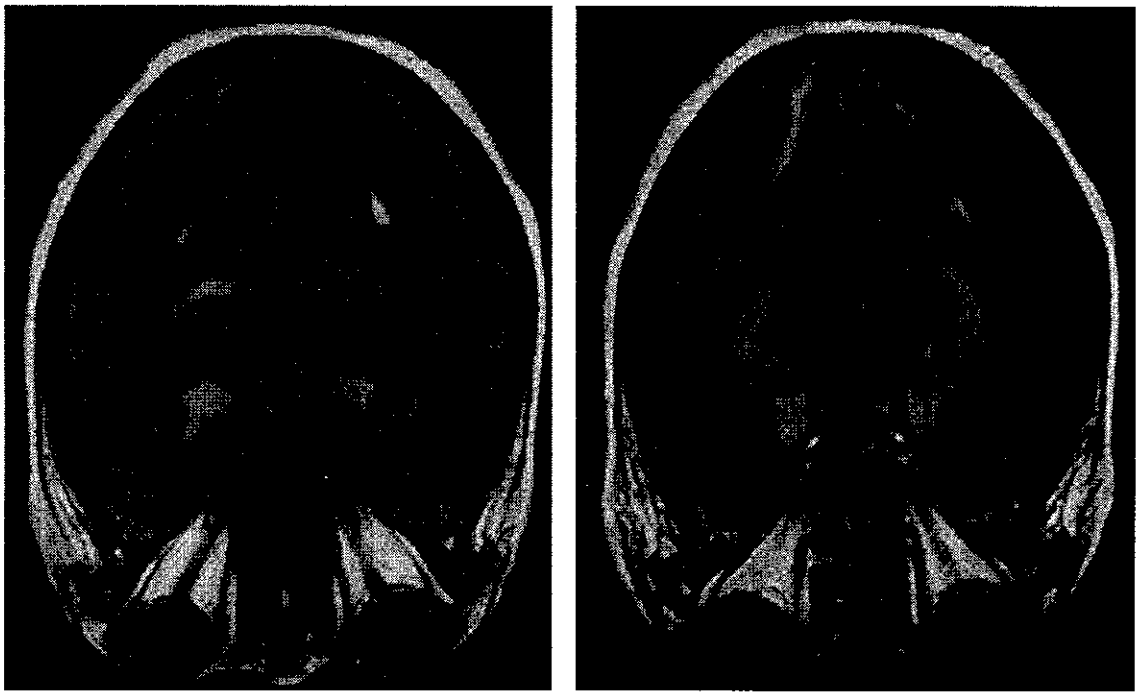

Figure 1. T2-weighted magnetic resonance image of the brain showing high-intensity signals in bilateral temporal lobes. The patient was diagnosed with limbic encephalopathy.

In contrast to conventional allo-HSCT, the incidence of metabolic encephalopathy is increased with RIST. In this study, 15 of 18 CNS complications were metabolic. Of these patients, 4 were diagnosed with cyclosporine encephalopathy on the basis of typical clinical and imaging findings. The incidence of cyclosporine encephalopathy was 1.7% after RIST, which is comparable to that after conventional allo-HSCT in young patients [24]. The median onset was 15 days (range, days 7-22). Three patients displayed seizures and altered mental status that improved after discontinuation of cyclosporine. Blood levels for cyclosporine were normal in all of the 4 patients. Risk factors for cyclosporine encephalopathy have been reported [24,25], and hypertension (2/4), hypocholesterolemia (1/2), and hypomagnesemia (3/4) were observed in our study. These findings are comparable to previous reports on cyclosporine neurotoxicity [24,25]. The growing use of RIST has increased the chance of cyclosporine being administered to elderly patients. Our study does not support the hypothesis that cyclosporine neurotoxicity increases in elderly patients, but further investigation of the safety issues for cyclosporine is warranted. General management such as blood pressure control and electrolyte replacement may be important in preventing adverse effects of cyclosporine.

No findings in the remaining 11 patients with metabolic encephalopathy suggested cyclosporine encephalopathy. However, it should be noted that all 11 patients received a fludarabine-based preparative regimen and that fludarabine has a considerable neurotoxicity [26–32]. These findings suggest that fludarabine might have contributed to the development of CNS toxicity in this study. Except for 1 patient with leukoencephalopathy and hemophagocytic syndrome–related CNS complications, the other 10 patients had undergone UCBT. The incidence of CNS complications after RI-UCBT was 24%. Cord blood as a stem cell source was an independent risk factor in multivariate analysis (odds ratio, 14.5; 95% confidence interval, 3.7-56.9; $P < .0001$). Few studies on CNS complications after myeloablative UCBT have been reported. This complication is possibly characteristic of RI-UCBT. All 10 patients developed altered mental status, including 3 with generalized seizures. Brain imaging in 3 patients showed abnormal signals around the hippocampus, whereas images were normal in the other 6 patients. Hippocampal encephalopathy in the 3 patients involved both white and gray matter and was thus distinct from leukoencephalopathy. Similar findings after RI-UCBT have recently been reported [33]. Although an association with tacrolimus administration has been suggested, none of our patients received tacrolimus, thus indicating other causes. Possibilities include infection, regimen-related toxicity, and immune reaction associated with the use of cord blood. Eight patients who developed metabolic encephalopathy after RI-UCBT had received fludarabine, melphalan, and TBI as a preparative regimen.

This has a higher intensity than most reduced-intensity regimens and might have caused CNS toxicities.

Conversely, CNS complications do not represent a significant concern in bone marrow or peripheral blood transplantation with similar reduced-intensity regimens. Because adult RI-UCBT recipients receive a relatively low dose of $CD34^+$ cells, it would raise the concern that there might have been delayed engraftment, leading to an increase in subclinical or undetected CNS viral infections. However, this possibility seemed unlikely. In RI-UCBT with fludarabine, melphalan, and intermediate-dose TBI as a preparative regimen and cyclosporine as GVHD prophylaxis [34], the median day of neutrophil engraftment was 17.5 days. This is comparable to RIST with granulocyte colony-stimulating factor–mobilized blood [11,13]. Furthermore, neither cerebrospinal findings nor blood cultures identified CNS infection in our study, and no patient had GVHD at the onset of CNS complications. Because 4 of the 10 patients who underwent RI-UCBT died soon after the development of CNS complications, symptoms might represent an early manifestation of a systemic disorder predisposing for multiple organ dysfunction syndrome, increasing the risk of transplant-related mortality [35]. However, the association of CNS complications with engraftment is noteworthy in RI-UCBT. We did not use antithymocyte globulin or corticosteroids for preparative regimens or GVHD prophylaxis, respectively, although these practices have been commonly used in previous studies on UCBT [36]. Both agents display strong immunosuppressive properties. The fluid accumulation often observed during this period may have accentuated the tendency for brain edema to develop, as seen in patients with renal decompensation. In RI-UCBT with our regimens [34], the cumulative incidence of complete donor chimerism at day 60 was 93%, and the median time to complete donor chimerism was 22 days. Grade II to IV acute GVHD occurred in 27% of patients. Approximately 60% of RI-UCBT recipients had a noninfectious fever before engraftment (median onset, day 9). Manifestations included a high-grade fever, eruption, and diarrhea, and corticosteroids were effective for ameliorating these reactions. These findings suggest that they might be associated with a cytokine storm induced by massive proliferation of cells with a unique cytokine profile and that the CNS toxicity was attributable to these immune responses. We therefore treated the CNS toxicity with corticosteroids. Because CNS toxicity is associated with considerable morbidity and mortality, optimal preventive measures for CNS complications after RI-UCBT should be established. Intensification of GVHD prophylaxis, such as with methotrexate, might prove beneficial for this purpose.

This investigation was a retrospective study based on medical records. Pathologic examinations were not used in most patients, and diagnosis of CNS complications was established on the basis of clinical and radiologic findings. Mild neurotoxicity associated with allo-HSCT was likely neglected, and incidences might have been underestimated in this study. Compared with autopsy studies, approximately half of the patients with neurologic complications had been diagnosed during life [4]. Further prospective evaluation is warranted to clarify incidences and clinical characteristics for CNS complications after RIST and to establish optimal preventive and therapeutic measures.

In conclusion, we have demonstrated that CNS complications are a common and frequently fatal complication after RIST, particularly after the use of umbilical cord blood. Metabolic encephalopathy is the most common subtype of CNS complication after RIST, and it frequently manifests as limbic encephalopathy in RIST with umbilical cord blood.

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Disseminated tuberculosis following reduced-intensity cord blood transplantation for adult patients with hematological diseases

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Summary:

Allogeneic hematopoietic stem cell transplantation (allo-SCT) recipients are prone to infections. The incidences of mycobacterial infections after allo-SCT in several case series vary from less than 0.1–5.5%. However, no study has been published on tuberculosis following unrelated cord blood transplantation (UCBT). We retrospectively reviewed medical records of 113 adult patients with a median age of 54 years who underwent reduced-intensity UCBT (RI-UCBT) at Toranomon Hospital from March 2002 to May 2004. *Mycobacterium tuberculosis* infections were diagnosed in three patients (2.7%), of these two patients developed primary infection and one patient developed reactivation of latent tuberculosis. The interval between RI-UCBT and the diagnosis of tuberculosis was 34, 41 and 61 days. All the patients had disseminated disease at diagnosis. Histological examination showed the lack of granuloma in caseous necrosis. Combination antituberculous treatments showed limited efficacy, and two patients died immediately after diagnosis. *M. tuberculosis* caused life-threatening illness, rapidly progressing in RI-UCBT recipients. The lack of granuloma in caseous necrosis suggests the impaired T-cell function in early post transplant phase of RI-UCBT. We should consider *M. tuberculosis* in the differential diagnoses of fever of unknown source after RI-UCBT.

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Unrelated cord blood transplantation (UCBT) is an attractive alternative for patients with hematologic malignancies who do not have a matched related or unrelated donor. Myeloablative UCBT for adult patients achieves engraftment in 90% of the cases and is associated with 15% of transplant-related mortality, mostly attributable to infection.[1] The feasibility of UCBT using reduced-intensity regimens (RI-UCBT) for adult patients with hematologic diseases has been reported.[2–4] Studies on immune recovery following UCBT suggested that RI-UCBT recipients may have reduced incidences of graft-versus-host disease (GVHD) and infectious complications.[5] However, little information is available on infections following RI-UCBT as well as myeloablative UCBT.[6–8]

Mycobacterium tuberculosis (*M. tuberculosis*) is a common pathogen in the world. It is endemic to East Asia including Japan. Japan conducts a nationwide program of *Bacillus Calmette-Guerin* (BCG) vaccination in infants and revaccination in school children at the age of 6, 7, 13 and 14 years since 1951 and 1954, respectively. Annual incidence of tuberculosis decreased from 698 per 100 000 in 1951 to 31 per 100 000 in 2000. Tuberculosis mostly affects people at the age of 60 years or older; 82% of the patients are 40 years or older.[9,10] Reactivation of latent tuberculosis is common in immunodeficient patients such as AIDS patients and organ transplant recipients.[11,12] Recent studies on tuberculosis following allogeneic hematopoietic stem cell transplantation (allo-SCT) have demonstrated that this is a significant problem in endemic countries with an incidence of 0.1–5.5%.[13–21]

This is the first report, to our knowledge, on tuberculosis following RI-UCBT. Detailed description of this complication would be informative in the management of RI-UCBT.

Patients and methods

Data collection

Medical records of 113 recipients who underwent RI-UCBT between March 2002 and May 2004 at Toranomon Hospital were reviewed for the diagnosis of tuberculosis. Their characteristics were shown in Table 1. All the patients

Table 1 Patients' characteristics and transplantation procedures

Variables	*Number*
Patients characteristics	
Age (median, range)	54 (17–79)
Sex (male/female)	65/48
Primary diseases	
AML/MDS	46
Malignant lymphoma	41
Acute lymphoblastic leukemia	15
Severe aplastic anemia	5
Chronic myeloid leukemia	1
Plasma cell neoplasms	4
Others	1
Risk of underlying diseases[a] (high/low)	108/5
Transplantation procedures	
GVHD prophylaxis (cyclosporine/tacrolimus)	89/24
Number of infused CD34+cells ($\times 10^7$/kg) (median, range)	2.9 (1.7–5.2)
HLA disparity (0/1/2)	2/15/96

[a]We divided the risk of transplantation into two groups. The low-risk group was as follows: acute myeloid or lymphoid leukemia in first and second remission, chronic myelogeneous leukemia in chronic phase and myelodysplastic syndrome refractory anemia. The other patients were defined as having high-risk diseases.
TBI = total body irradiation; AML = acute myeloid leukemia; MDS = myelodysplastic syndromes.

had received BCG vaccines according to the national vaccination program.[10] No patients had undergone anti-tuberculosis treatments prior to RI-UCBT.

The following data of the patients with tuberculosis were collected: demographics, past medical history including tuberculosis, the primary hematologic diseases, the presence of acute or chronic GVHD, chest radiographs, diagnostic methods and the primary site of *M. tuberculosis* infection, and the outcome of tuberculosis treatment.

Transplantation procedures and supportive care

The pretransplant evaluation of the respiratory system included chest radiographs, high-resolution computed tomographies (CT) and pulmonary function tests. Sputum smears and cultures for acid-fast bacilli were not routinely obtained.

A cord blood (CB) unit was searched through the Japan Cord Blood Bank Network. CB units, which were available for UCBT were 4/6 or more serologically HLA-antigen matched and contained at least 2×10^7 nucleated cells/kg of recipient body weight before freezing.

Transplantation procedures were shown in Table 1. The RI conditioning regimen consisted of fludarabine 25 mg/m^2 on days −6 to −2, melphalan 40 mg/m^2 on days −3 and −2, and total body irradiation (TBI) 4 Gy on day −1. Granulocyte colony-stimulating factor was administered from day 1 until neutrophil engraftment.

GVHD prophylaxis was either tacrolimus 0.03 mg/kg or cyclosporine 3 mg/kg in a continuous infusion starting on day −1. The diagnosis of GVHD was made based on clinical judgment and skin or gut biopsy results to support the clinical diagnosis. Acute GVHD was graded according to the consensus criteria.[22] When patients developed grade II–IV acute GVHD, we initiated 1–2 mg/kg/day of methylprednisolone in addition to tacrolimus or cyclosporine.

Patients were managed in reverse isolation in laminar airflow-equipped rooms. All patients received prophylaxis with trimethoprim-sulfamethoxazole against *Pneumocystis carinii* infection. They received tosufloxacine 450 mg/day, fluconazole 200 mg/day and acyclovir 600 mg/day for the prophylaxis of bacterial, fungal and herpesvirus infection, respectively. Broad-spectrum antibiotics were initiated when neutropenic fever developed. All patients were monitored for cytomegalovirus pp65 antigenemia once a week. When the antigenemia turned positive, pre-emptive therapy with foscarnet was initiated as described previously.[23]

Definition of M. tuberculosis *infection*

A diagnosis of tuberculosis was based on the identification of *M. tuberculosis* on acid-fast bacilli stain of sputum, endotracheal aspirates, bone marrow aspirates or bronchoalveolar lavage (BAL), or the presence of caseous granulomas on hematoxylin–eosin staining of tissue specimens.[24] All of the BAL specimens were subject to polymerase chain reactions (PCR) using *M. tuberculosis*-specific primers[25] and COBAS AMPLICOR™ system (Roche Diagnostics KK, Tokyo, Japan).

Disease control for tuberculosis

All the staff members were screened for tuberculosis by chest X-ray annually. Anyone with positive chest X-ray and suggestive symptoms are not on service.

Any patients with suspected tuberculosis are placed in respiratory isolation until three consecutive sputum samples are negative for acid-fast bacilli. Patients with the diagnosis of tuberculosis receive the standard antituberculosis treatment with rifampicin, isoniazid and ethambutol, with or without pyrazinamide. Since rifampicin may decrease cyclosporine concentration, cyclosporine dose was adjusted by monitoring its blood levels.

Results

Three of the 113 RI-UCBT recipients (2.7%) developed tuberculosis. They had no different risk factors for tuberculosis compared with the other 110 patients. Their detailed clinical characteristics are shown in Table 2.

Patient 1

A 67-year-old man with chronic myeloid leukemia in blastic crisis underwent RI-UCBT in July 2003. He had no family history of tuberculosis. Screening chest CT before transplantation was normal. He developed a pre-engraftment immune reaction on day 9,[3] which was successfully treated by methylprednisolone 0.25 mg/kg. He achieved neutrophil engraftment on day 19. He developed grade II acute GVHD on day 24, which responded to methylprednisolone. He developed a high-grade fever on day 61, and chest CT

Table 2 Clinical features of three patients who developed disseminated tuberculosis following RI-UCBT

	Patient 1	*Patient 2*	*Patient 3*
Primary disease	CML	AML	AML
Disease status	Blastic crisis	Primary induction failure	Second remission
Age (years)/sex	67/male	64/female	20/female
Past and family history of tuberculosis	None/none	None/none	None/none
Screening chest CT scan	Normal	Small calcified nodules in right lower lobe	Normal
Pre-engraftment immune reaction	Present	Present	Absent
Maximal grade of acute GVHD	Grade I	Absent	Absent
Corticosteroid use	Yes	Yes	No
Onset of tuberculosis	Day 63	Day 34	Day 46
Initial symptoms	Fever, fatigue	Fever, cough, fatigue	Fever, chest pain
Chest CT finding at diagnosis	Multiple cavitations	Infiltration in the right lower lobe, wall thickening of bronchus	Mediastinal lymphadenopathy
Type of tuberculosis	Miliary tuberculosis	Miliary tuberculosis	Miliary tuberculosis
Treatment	INH/RFP/EB/PZA	INH/RFP/EB	INH/RFP/EB
Outcomes	Died of miliary tuberculosis on day 116	Died of miliary tuberculosis on day 57	Improved, alive on day 180

CML = chronic myeloid leukemia; AML = acute myeloid leukemia; INH = isoniazid; RFP = rifampicin; EB = ethambutol; PZA = pyrazinamide.

on day 62 revealed small nodules in bilateral lungs, which were 2 mm in diameter. The lesions were considered to be bacterial or fungal infection and we initiated empiric treatments. The fever persisted despite broad-spectrum antibiotics and antifungal agents. Follow-up CT on day 75 showed multiple cavities in bilateral lungs, which were 12 mm in diameter (Figure 1a). Ziehl–Neelsen stain and PCR of BAL specimens on day 77 were positive for *M. tuberculosis*. Subsequently, *M. tuberculosis* was cultured from BAL fluid and bone marrow aspirates. On day 61, white blood cell count was 7.3×10^9/l (neutrophil 91.0%) with 95 CD4 + T-cells/μl; serum IgG was 306 mg/dl. Cyclosporine was tapered and a combination therapy with isoniazid, rifampicin, ethambutol and pyrazinamide was initiated. GVHD aggravated after tapering of cyclosporine, which was successfully treated with methylprednisolone 1 mg/kg. On day 89, he developed respiratory failure due to interstitial pneumonitis, requiring mechanical ventilation. He died of multiple-organ failure on day 116. In the post-mortem examination, almost all organs showed necrosis without granulation (Figure 2a). Ziehl–Neelsen stain identified acid-fast bacilli in the necrosis.

Patient 2

A 64-year-old woman with refractory acute myeloid leukemia underwent RI-UCBT in August 2003. She had no history of tuberculosis. Screening chest CT showed a small calcification nodule in the right lower lobe, which was 10 mm in diameter. She developed a pre-engraftment immune reaction on day 9,[3] which was successfully controlled by methylprednisolone 0.25 mg/kg. She achieved neutrophil engraftment on day 23. The clinical course had been uneventful until day 34, when a high-grade fever developed. Chest CT on day 34 showed infiltration in the right lower lobe, thickening of bronchial wall and pericardial effusion (Figure 1b). The patient refused bronchoscopy at this point. The fever persisted despite broad-spectrum antibiotics and antifungal drugs. BAL specimens obtained on day 43 were positive for *M. tuberculosis* on PCR. The calcified nodule in the right lobe indicated reactivation of latent tuberculosis. Phenotype of the peripheral lymphocytes on day 43 was shown in Table 2. Combination therapy with isoniazid, rifampicin and ethambutol was immediately initiated. However, it was discontinued due to hepatic toxicity. She died of adult respiratory distress syndrome on day 57. In the post-mortem examination, almost all organs had necrosis without granulation, and Ziehl–Neelsen stain revealed acid-fast bacilli in the necrotic lesions (Figure 2b). She also had disseminated cytomegalovirus infection.

Patient 3

A 20-year-old woman with acute myeloid leukemia in second remission underwent RI-UCBT in November 2003. Screening chest CT was normal. She achieved neutrophil engraftment on day 28. The clinical course had been uneventful until she developed a high-grade fever on day 46. She had not developed either a pre-engraftment fever or acute GVHD.[3] Chest CT showed mediastinal lymphadenopathy, which was 15 mm in diameter (Figure 1c). The fever did not respond to empiric broad-spectrum antibiotics. Bone marrow aspirates on day 65 were positive for *M. tuberculosis* on PCR and culture. She subsequently developed cutaneous tuberculosis (Figure 3) with disseminated intravascular coagulation. She was diagnosed with primary infection of *M. tuberculosis*. On day 46, white blood cell count and serum levels of IgG were 5.6×10^9/l (neutrophil 84.5%) and 1180 mg/dl, respectively. Phenotype of the peripheral lymphocytes on day 46 was shown in Table 2. After initiation of isoniazid, rifampicin and ethambutol, these symptoms improved gradually. She is alive without reactivation of tuberculosis on day 180.

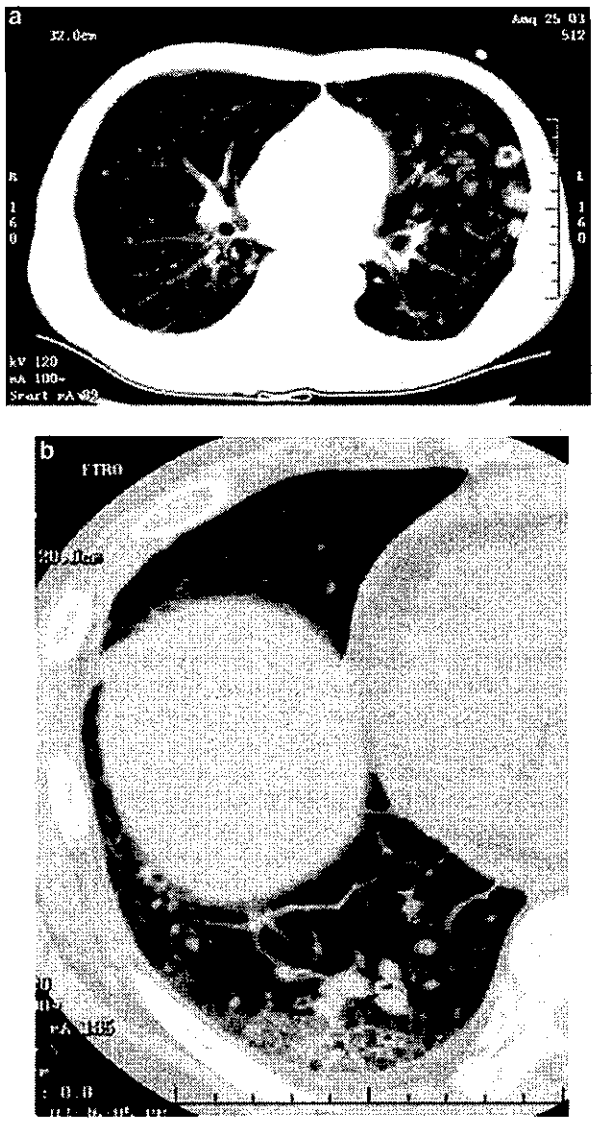

Figure 1 Chest CT scans. (a) Patient 1: follow-up CT on day 75 showed multiple cavities in bilateral lungs, which were 12 mm in diameter. (b) Patient 2: chest CT on day 34 showed infiltration in the right lower lobe, thickening of bronchial wall and pericardial effusion. (c) Patient 3: enlarged mediastinal lymph nodes.

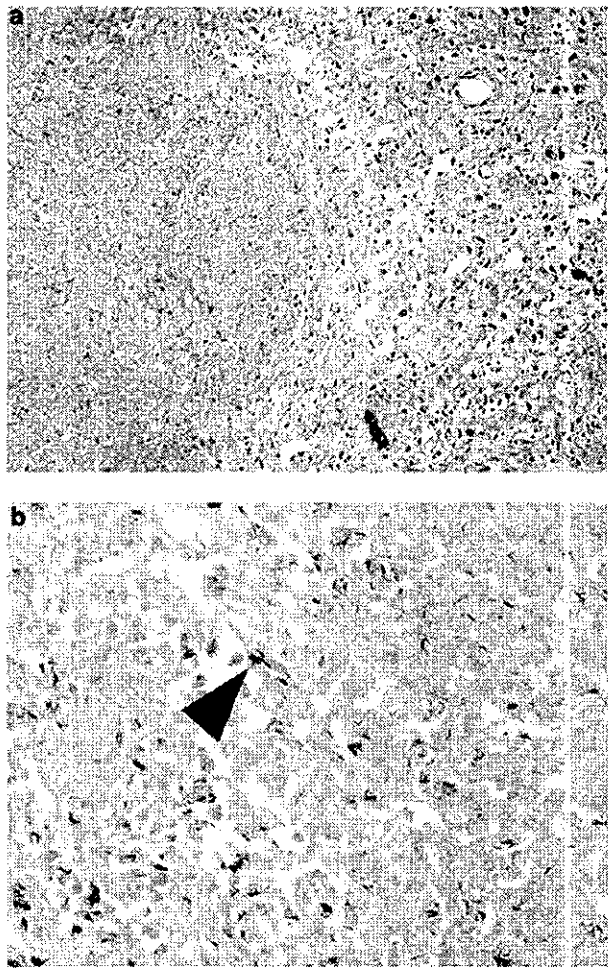

Figure 2 Pathologic features of tuberculosis infection. (a) Caseous necrosis without granulation in the lung. Paraffin section with hematoxylin–eosin stain; original magnification × 100. (b) Acid-fast bacilli (arrowhead) in caseous necrosis in the liver. Paraffin section with Ziehl–Neelsen stain; original magnification × 400.

Discussion

Japan has been an endemic country of tuberculosis. Among the newly diagnosed patients in 2001, 57.1% were at the age of 60 years and above and 17.5% were in their 20's and 30's. Many patients above 50 have a history of tuberculosis. Even without obvious history of tuberculosis, latent infection after unknown exposure is common in the generation. Therefore, Patients 1 and 2 were considered to have reactivation of latent tuberculosis. Transmission from them to Patient 3 was unlikely because Patient 3 was admitted to the hospital 20 and 25 days after patients 1 and 2 had died, respectively. No patients had tuberculosis during her hospitalization. Owing to her young age, her tuberculosis was considered a primary infection rather than reactivation of latent infection.

The incidence of tuberculosis following RI-UCBT is 2.5% in our hospital, and the median age was 54 years in this study. A high incidence of tuberculosis might be

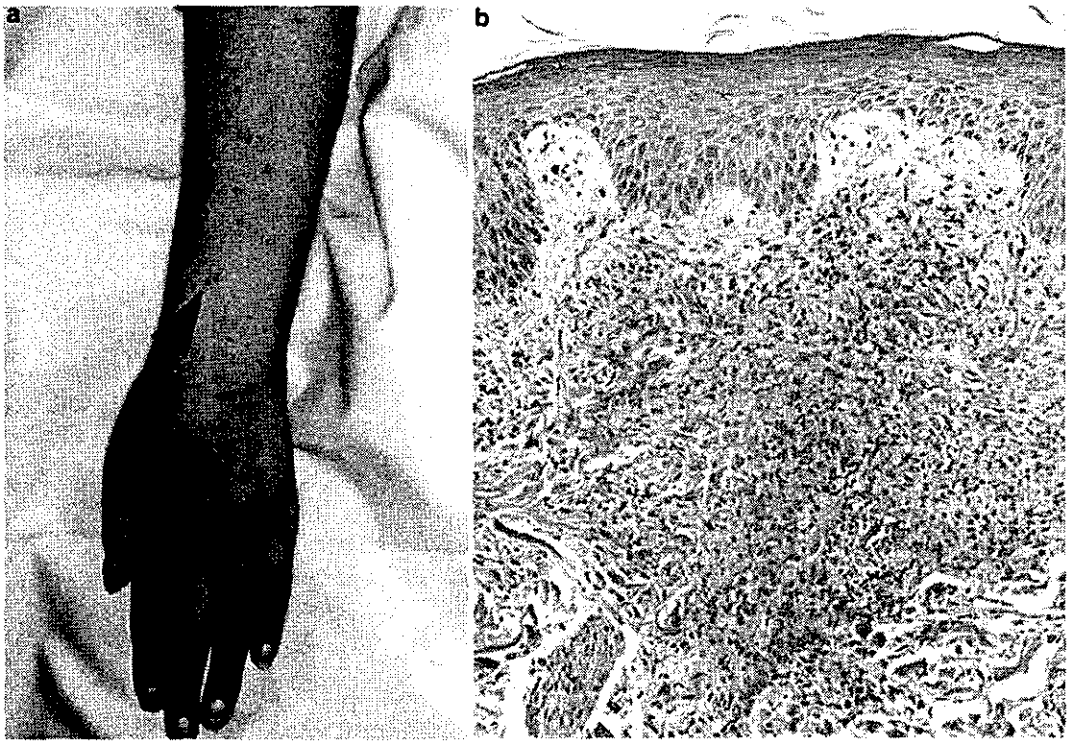

Figure 3 Macroscopic and pathologic features of cutaneous tuberculosis. (a) Disseminated tuberculosis (arrowhead) in the forearm. (b) Caseous necrosis and granuloma in the dermis. Paraffin section with hematoxylin–eosin stain; original magnification × 100.

expected in RI-UCBT patients due to patients' age and prolonged immunosuppression associated with the use of CB as a stem cell source; however, the incidence of tuberculosis following RI-UCBT was comparable to that following allo-HSCT in previous reports from endemic countries.[15,18–21] Tuberculosis is less frequent in RI-UCBT recipients than in the immunodeficient patients of different etiologies such as solid organ transplantation and AIDS. Immunosuppression after RI-UCBT is transient,[11] while AIDS patients and solid organ transplant recipients have a life-long immunosuppression. Alternatively, the frequent and prolonged use of several antibiotics may suppress reactivation of *M. tuberculosis*. Fluoroquinolones are active against *M. tuberculosis*, and are occasionally used as antituberculous prophylaxis.[16]

Clinical features of tuberculosis following RI-UCBT might be different from those following conventional allo-HSCT.[13–21] Tuberculosis following conventional allo-HSCT usually occurs several months after transplantation; the median time to presentation with tuberculosis was 324 days post transplant.[18] It usually follows indolent clinical courses. The lung is the most common site of the disease, and the common manifestations are fever and cough. If treated adequately, most patients are cured without relapse. The clinical courses of our patients were different from those in the previous reports.[13–21] Tuberculosis developed within 100 days of RI-UCBT, either reactivation or primary infection, and disseminated rapidly to various organs. It should be noted that all the three patients developed miliary tuberculosis, and two of the three patients were refractory to antituberculosis therapy and finally died. Such a rapid extrapulmonary progression of tuberculosis has been reported in solid organ recipients or AIDS patients.[26,27]

Table 3 Phenotypic analysis of peripheral lymphocytes at the onset of tuberculosis

	Patient 2	*Patient 3*
White blood cells ($\times 10^9$/l)	2.0	1.5
Lymphocyte ($\times 10^9$/l)	0.28	0.12
CD4+T cell ($\times 10^9$/l)	0.09	0.04
CD8+T cell ($\times 10^9$/l)	0.07	0.034
CD4/8 ratio	1.24	1.14
CD4+, 45RA+ ($\times 10^9$/l)	0.044	0.011
CD4+, CD45RO+ ($\times 10^9$/l)	0.01	0.022
CD4+, CD69+ ($\times 10^9$/l)	0.0	0.0011
CD4+, CD25+ ($\times 10^9$/l)	0.06	0.021
CD20+ ($\times 10^9$/l)	0.004	0.001
CD3−, CD56+ ($\times 10^9$/l)	0.08	0.019

Although limited data are available on immune reconstitution of RI-UCBT recipients, it is clear their cellular immunity is extremely impaired (Table 3). While recovery of the immune system depends on peripheral expansion of mature T- and B-lymphocytes transferred with the graft,[28] CB does not contain antigen-experienced cells, leading to a slow immune reconstitution and an increased risk of infectious complications. Functional immune recovery begins 3 months after conventional UCBT in adult patients, and T-cell reconstitution begins by 18 months.[29]

Chronic GVHD, immunosuppressive therapy, TBI and T-cell depletion have been shown to be risk factors for tuberculosis following conventional allo-HSCT.[16,17,30] We used a TBI-containing preparative regimen, and two patients developed either GVHD or pre-engraftment immune reaction, requiring corticosteroid. Prolonged immunosuppression results in failure to acquire adoptive immunity against tuberculosis, and TBI hampers normal function of alveolar macrophages. Both might have contributed to an increased susceptibility to early-onset tuberculosis.

This study demonstrates that tuberculosis is a significant complication of RI-UCBT. There is a considerable delay from the onset of symptoms to the diagnosis of tuberculosis. Unless we have enough information on the complication, it is difficult to make an early diagnosis and to initiate an antituberculous treatment. A delay in establishing a diagnosis of tuberculosis in RI-UCBT recipients could have a pivotal impact on their survival. While needs for antituberculous prophylaxis are controversial in solid organ and marrow transplantation,[31] identification of high-risk patients and prophylaxis in the subgroup may be beneficial in RI-UCBT. We suggest screening before RI-UCBT with PPD skin test in addition to chest CT, especially in the endemic areas. Further studies are warranted to investigate clinical features of tuberculosis following RI-UCBT, and to identify its optimal management.

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Tyrosine Kinase 2 Interacts with and Phosphorylates Receptor for Activated C Kinase-1, a WD Motif-Containing Protein[1]

Takashi Haro,* Kazuya Shimoda,[2]* Haruko Kakumitsu,* Kenjirou Kamezaki,* Akihiko Numata,* Fumihiko Ishikawa,* Yuichi Sekine,† Ryuta Muromoto,† Tadashi Matsuda,† and Mine Harada*

Receptor for activated C kinase (Rack)-1 is a protein kinase C-interacting protein, and contains a WD repeat but has no enzymatic activity. In addition to protein kinase C, Rack-1 also binds to Src, phospholipase Cγ, and *ras*-GTPase-activating proteins. Thus, Rack-1 is thought to function as a scaffold protein that recruits specific signaling elements. In a cytokine signaling cascade, Rack-1 has been reported to interact with the IFN-αβ receptor and Stat1. In addition, we show here that Rack-1 associates with a member of Jak, tyrosine kinase 2 (Tyk2). Rack-1 interacts weakly with the kinase domain and interacts strongly with the pseudokinase domain of Tyk2. Rack-1 associates with Tyk2 via two regions, one in the N terminus and one in the middle portion (aa 138–203) of Rack-1. Jak activation causes the phosphorylation of tyrosine 194 on Rack-1. After phosphorylation, Rack-1 is translocated toward the perinuclear region. In addition to functioning as a scaffolding protein, these results raise the possibility that Rack-1 functions as a signaling molecule in cytokine signaling cascades. *The Journal of Immunology,* **2004, 173: 1151–1157.**

Many cytokines bind to specific cell surface receptors and activate members of the Janus family of protein tyrosine kinases (Jaks), which are associated with cytokine receptors (1). The activated Jaks phosphorylate the tyrosine residues of the receptors, thereby recruiting Stats and other signaling molecules into the activated receptor complex. Stats are then phosphorylated by Jaks, and are subsequently translocated to the nucleus, where they affect gene expression. This Jak-Stat signaling pathway is widely used by members of the cytokine receptor super family (2). There are four mammalian Jaks: Jak1, Jak2, Jak3, and tyrosine kinase 2 (Tyk2).[3] Tyk2 has been identified as a novel protein kinase which can compensate for IFN-α nonresponsive mutated fibroblasts (3). IFN-α specifically activates Jak1 and Tyk2, which phosphorylate Stat 1 and 2. These activated Stats subsequently associate to form either Stat1 homodimers or the transcription factor ISGF-3, which then translocate to the nucleus to regulate gene expression (2, 4). Both Tyk2 and Jak1 were thought to be essential for signal transduction downstream of IFN-α in mutated fibroblasts, which are not responsive to IFN-α (4). However, using Tyk2-deficient mice, we and others have shown that Tyk2 has a restricted function and does not play a major role in IFN-α signaling (5, 6). In contrast, Jak 1-null cells fail to respond to IFN-α (7). In addition, Stat1-null mice are defective in almost all IFN-α-induced responses (8, 9). Stat2-null mice also demonstrated an increased susceptibility to viral infection (10). Based on these data, the Jak1-Stat signaling pathway is thought to be essential for IFN-α signaling. Recently, we reported that Tyk2 was essential for IFN-α-induced B lymphocyte growth inhibition (11). Stat1 is not required for this IFN-α-mediated inhibition (12); therefore, other signaling molecules must exist downstream of activated Tyk2 to transduce the IFN-α signal inhibiting B lymphocyte growth. Thus, we performed a yeast two-hybrid screen for proteins that interact with Tyk2.

In this report, we identify receptor for activated C kinase (Rack)-1, originally described as a receptor for activated C kinase β, as a protein that interacts with Tyk2. Rack-1 has previously been reported to constitutively interact with the β long subunit of the type I IFNR (13), Jak1, Tyk2 (14), and Stat1 (15). We show here that Rack-1 associates with Jaks, and is phosphorylated on tyrosine residues by Jaks. This raises the possibility that Rack-1 functions as a signaling molecule in cytokine signaling cascades.

Materials and Methods

Abs and reagents

Anti-Rack-1, -Tyk2, -Jak1, -Jak2, and -Jak3 Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine mAb (clone 4G10) was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-c-Myc mAb was purchased from BD Clontech (Palo Alto, CA). Anti-Flag M2 mAb was purchased from Sigma-Aldrich (St. Louis, MO). Murine IFN-α was purchased from HyCult Biotechnology (Uden, The Netherlands).

Yeast two-hybrid screen

A cDNA encoding the kinase domain (aa 833-1187) of human Tyk2 was subcloned into the Gal DNA-binding domain plasmid pGBKT7 (BD Clontech), and was used as bait in a yeast two-hybrid screen of a human B lymphocyte cDNA library constructed in pACT (BD Clontech). Approximately 1.6×10^6 colonies were screened for activation of the ADE2, HIS3, and *lacZ* reporter genes using the host strain AH109. The inserts from positive library plasmids were then amplified by PCR and mapped by *AluI* digestion. Plasmids were sequenced after isolation and bacterial transformation.

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[3] Abbreviations used in this paper: Tyk2, tyrosine kinase 2; Rack, receptor for activated C kinase; PKC, protein kinase C.

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Mammalian expression vector constructs

Murine Jak1, Jak2, Jak3, and human Tyk2 expression constructs were gifts from Dr. J. Ihle (St. Jude Children's Research Hospital, Memphis, TN). Kinase-negative versions of the Jak proteins were generated by mutating lysine (K833) to glutamine in murine Jak1 (Jak1 KE) and lysine (K882) to glutamine in murine Jak2 (Jak2 KE) (16).

The partial human Tyk2 cDNAs expressing the various domains (aa 1–450, 266–733, 600–1086, or 833–1187) indicated in Fig. 3*A* were generated by PCR, and were subcloned into pCMV-MyC (BD Clontech). The clone 4-86 cDNA (C-terminal region of Rack-1 encoding aa 137–317), whose gene product associated with Tyk2 (aa 833–1187) in yeast, was removed from pACT2 by *SfiI/XhoI* digest, and was subcloned into pCMV-HA (BD Clontech). The full-length human Rack-1 cDNA was generated by RT-PCR from human peripheral blood lymphocytes, and was subcloned into pCR2.1-TOPO (Invitrogen Life Technologies, Carlsbad, CA). *HindIII-ApaI*, *XhoI*, *BamHI-EcoRI*, *BglII-EcoRI*, and *BamHI* fragments of the full-length Rack-1 were subcloned into the Flag-tagged mammalian expression plasmid pCMV-Tag2 (BD Clontech) to generate Rack-1 (aa 1–317), (aa125–317), (aa 204–317), (aa 258–317), and (aa 1–204) indicated in Fig. 3*C*. Rack-1 (1–137) was generated by PCR using Rack-1 in pCR2.1-TOPO as the template, and was subcloned into the *BamHI* site in pCMV-Tag2. Rack-1 (Δ138–203) was generated by ligation of the aa 1–137 fragment to the 204–317 fragment in pCMV-Tag2.

Oligonucleotide-directed mutagenesis was used to substitute phenylalanine for tyrosine at residues 140, 194, 228, and 246 of wild-type Rack1 in pCMV-Tag2. The Transformer site-directed mutagenesis kit was used according to the manufacturer's protocol (BD Clontech) with the following oligonucleotides: Y140F oligo, GGTGTGTGCAAATTCACTGTCCAG; Y194F oligo, CACACAGGCTTTCTGAACACGGTG; Y228F oligo, GGCAAAC ACCTTTTCACGCTAGAT; Y246F oligo, CCTAACCGCTTCTGGCTGT GTGCT.

Cell culture and transfection

HEK293T cells were plated at 2–4 × 10^6 cells/ml in DMEM (Sigma-Aldrich) containing 10% heat-inactivated FBS (JRH Biosciences, Lenexa, KS), 2 mM L-glutamine (Invitrogen Life Technologies; Gaithersburg, MD), MEM nonessential amino acids (Invitrogen Life Technologies), 1 mM sodium pyruvate (Invitrogen Life Technologies), 100 U/ml penicillin, and 10 μg/ml streptomycin (Invitrogen Life Technologies), and grown at 37°C in 5% CO_2 to 60% confluence. 293T cells were transfected with 10 μg of plasmid DNA using the calcium phosphate precipitation protocol. Cells were cultured to almost 100% confluence and harvested, after appropriate stimulation.

The IL-3-dependent cell line, BAF/3, was maintained in RPMI 1640 medium supplemented with 10% FCS and IL-3.

Immunoprecipitation and immunoblotting

Cells were lysed as previously described (16). Cell lysates were centrifuged at 12,000 × *g* for 15 min to remove debris. For immunoprecipitation, the indicated Abs were added to the supernatant of each sample, incubated for 8 h, and mixed with protein A-agarose (Sigma-Aldrich). Total cell lysates or immunoprecipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences, Uppsala, Sweden). Membranes were probed using the appropriate Abs and visualized by ECL (Amersham Biosciences).

Immunofluorescence

HEK293T cells were maintained in DMEM containing 10% FCS and transfected with Flag-tagged wild-type or E mutant Rack-1 with or without Tyk2, Jak1, Jak2, the Jak1 KE mutant, or the Jak2 KE mutant by the calcium phosphate precipitation protocol. Forty-eight hours after transfection, cells were fixed with a solution containing 4% paraformaldehyde and incubated with an anti-Flag Ab. The cells were then incubated with a FITC-conjugated anti-mouse IgG Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) and mounted with a drop of Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Cells were observed using a confocal laser fluorescence microscope. The intracellular localization of labeled Rack-1 was assessed in reference to nuclear 4′6′-diamidine-2-phenylindole dihydrochloride (DAPI) staining. The digital images were processed by layering and the contrast of all images was increased by 50% using Adobe Photoshop 4.0 (Adobe Systems, Mountain View, CA). Nuclear DAPI staining appears blue and Rack-1 appears green.

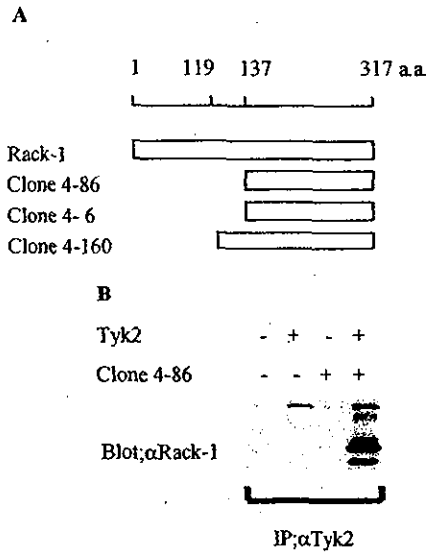

FIGURE 1. Rack-1 interacts with Tyk2. *A*, A schematic representation of the Rack-1 protein identified by yeast two-hybrid screen using the Tyk2 tyrosine kinase domain as bait. Amino acids 137–317 of Rack-1 were present in three clones which interacted with Tyk2 in yeast cells. *B*, Coimmunoprecipitation of clone 4-86 with Tyk2 in mammalian 293T cells. 293T cells were transfected with clone 4-86 and/or Tyk2. Cell lysates were immunoprecipitated with anti-Tyk2 Ab, and immunoblotted with anti-Rack-1 Ab.

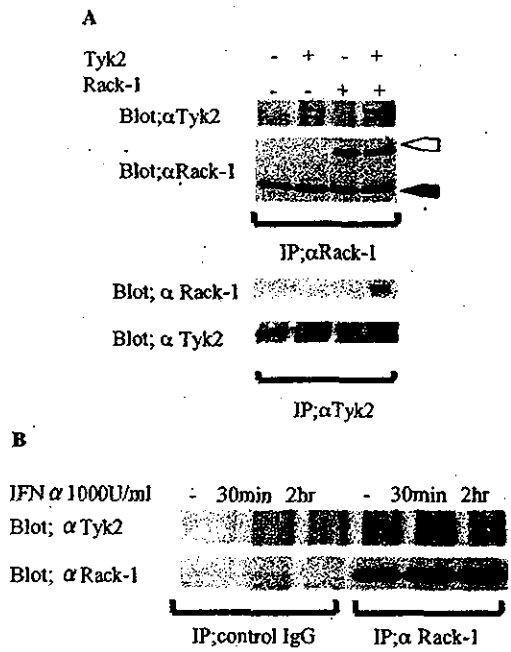

FIGURE 2. The physical interaction between Rack-1 and Tyk2 is independent of IFN stimulation. *A*, 293T cells were transfected with Tyk2 and/or Rack-1. *Top*, Cell lysates were immunoprecipitated with anti-Rack-1 Ab and immunoblotted with anti-Tyk2 (*upper panel*) or anti-Rack-1 Ab (*lower panel*). Open arrow indicates transfected Rack-1, and the filled arrow indicates endogenous Rack-1. *Bottom*, Cell lysates were immunoprecipitated with anti-Tyk2 Ab and immunoblotted with anti-Rack-1 Ab (*upper panel*) or anti-Tyk2 Ab (*lower panel*). *B*, BAF/3 cells were either stimulated for the indicated time with IFN-α (1000 U/ml) or left unstimulated as a control. Cell lysates were immunoprecipitated with nonspecific mouse IgG or anti-Rack-1 Ab and immunoblotted with anti-Tyk2 Ab (*upper panel*) or anti-Rack-1 Ab (*lower panel*).

Results

Identification of Rack-1 as a Tyk2-interacting protein

To identify novel Tyk2-interacting proteins, a yeast two-hybrid screen was performed using the kinase domain of Tyk2 fused to the Gal DNA-binding domain as bait. A human bone marrow cell cDNA library was screened. Among a number of positive clones, three clones termed 4-6, 4-86, and 4-160, encoded the C-terminal portion of the previously described Rack-1 (Fig. 1*A*). To determine whether these clones encoded a protein able to interact with Tyk2 in mammalian cells, clone 4-86 was subcloned into the mammalian expression vector pCMV-HA. The resulting expression vector produced a protein that interacted strongly with Tyk2 in 293T cells (Fig. 1*B*), suggesting that the region comprising aa 137–317 of Rack-1 contains the binding site for Tyk2.

We next examined the association between Tyk2 and Rack-1 using the full-length cDNA in mammalian cells. The full-length Flag tagged Rack-1 cDNA was transiently transfected into 293T cells with or without the full-length Tyk2 expression vector. Fig. 2*A* shows that Tyk2 coimmunoprecipitated with Rack-1 and Rack-1 coimmunoprecipitated with Tyk2 when both Tyk2 and Rack-1 were expressed in 293T cells. We next investigated whether endogenous Rack-1 was able to interact with Tyk2 and how this might be affected by the activation of Tyk2. To activate Tyk2, a mouse pro-B cell line, BAF/3, was stimulated or not stimulated with IFN-α. Tyk2 immunoprecipitates were assessed for the presence of endogenous Rack-1 by Western blotting. As can be seen in Fig. 2*B*, Tyk2 was present in the Rack-1 immunoprecipitates. Furthermore, equivalent amounts of Tyk2 were present in samples which had or had not been stimulated with IFN-α. This indicates that Rack-1 associates with Tyk2 in BAF/3 cells, and that this interaction is not altered by stimulation with IFN-α.

Mapping the sites in Tyk2 and Rack-1 required for binding

We next sought to determine which region of Tyk2 was responsible for the interaction with Rack-1. We separated the Tyk2 cDNA into four regions (Fig. 3*A*), and each region was subcloned in frame with a *myc* tag in the mammalian expression vector pCMV-Myc. The full-length Tyk2 construct or each partial Tyk2 construct was transiently transfected into 293T cells along with the full-length Rack-1 construct. Cell lysates were immunoprecipitated with anti-Tyk2 or anti-Myc Ab, and Western blotted with

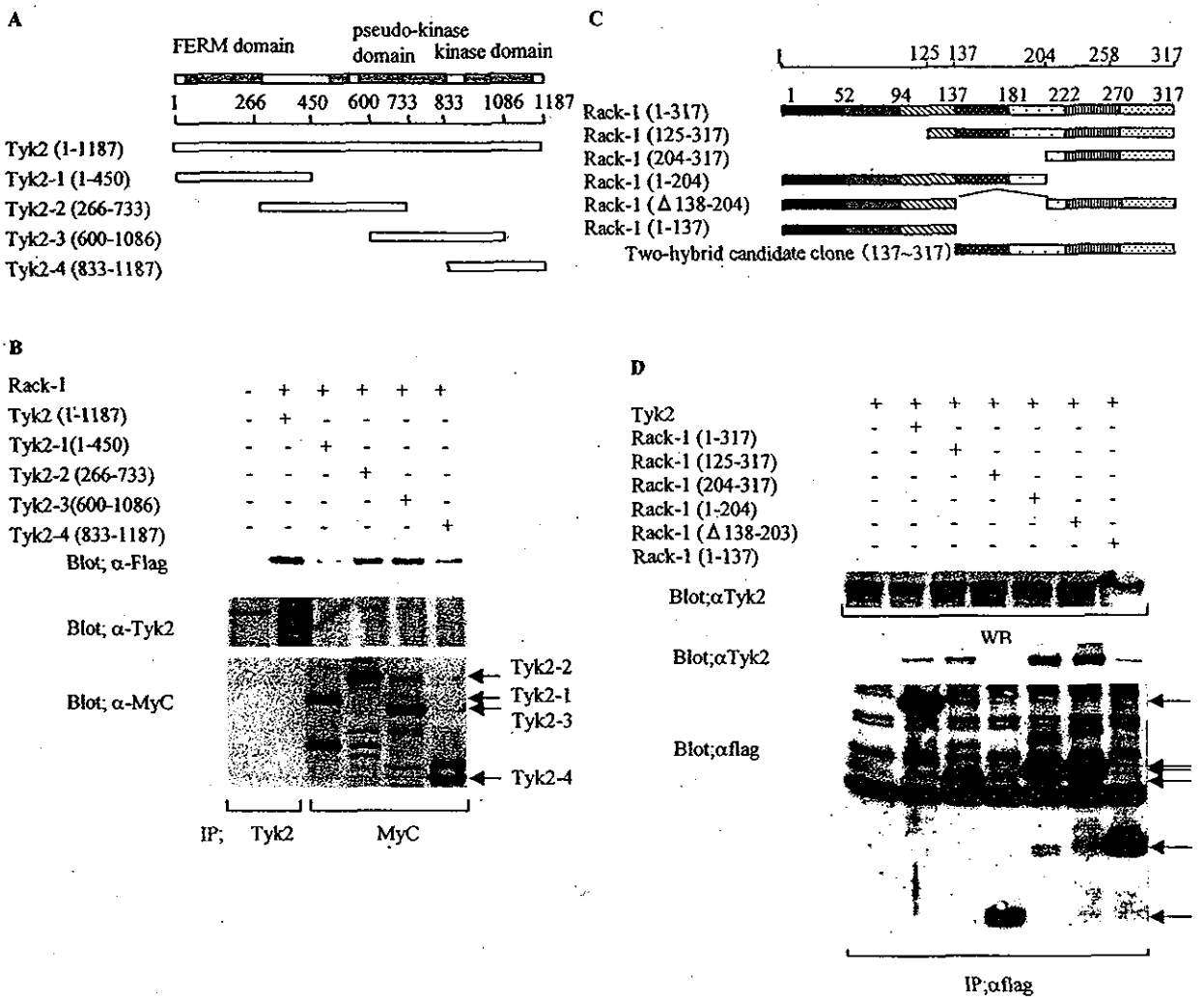

FIGURE 3. Mapping the sites in Tyk2 and Rack-1 required for binding. *A*, A schematic of the domain structure of Tyk2 and the mutant fragments. *B*, Full-length Tyk2 or Myc-tagged Tyk2 mutants were coexpressed with Flag-tagged Rack-1 in 293T cells. Forty-eight hours after transfection, the cells were lysed and immunoprecipitations were performed with anti-Tyk2 or anti-Myc Ab. The immunoprecipitates were immunoblotted with anti-Flag Ab (*upper panel*), anti-Tyk2 Ab (*middle panel*), or anti-Myc Ab (*lower panel*). *C*, A schematic of the domain structure of Rack-1 and the mutant fragments. *D*, Rack-1 mutants were coexpressed with Tyk2 in 293T cells. Forty-eight hours after transfection, the cells were lysed and immunoprecipitations were performed with anti-Tyk2 Ab. The immunoprecipitates were immunoblotted with anti-Flag Ab (*upper panel*) or anti-Tyk2 Ab (*lower panel*).