protocols for RQ-PCR amplification,[19] a commercial kit (Roche Diagnostics) or unpublished 'in-house' primers and probes (six laboratories).

Statistical method

For each vial and sample dilution, *ABL* and *BCR-ABL* CN were calculated using plasmid standard curves. The estimated CNs supplied by the participants were used. For each sample dilution, the geometric mean across replicate assays was calculated to give a single estimate of CN. The ratio *BCR-ABL* CN/*ABL* CN, also called normalized copy number (NCN), was expressed as a percentage.

Results

Comparison of lyophilized and fresh K562 cells for ABL, GUS and BCR-ABL expression

RNA extracted from both lyophilized and fresh K562 cells was shown to be of good quality, as determined by the Agilent 2100 bioanalyzer (Figure 1a and b). Table 1 shows results of RQ-PCR analysis, with mean CN values calculated from seven different RT reactions for *ABL*, *GUS* and *BCR-ABL*. Although some variation was observed between replicate experiments, no significant differences were observed in the relative levels of the three transcripts, between fresh and lyophilized preparations of K562 cells.

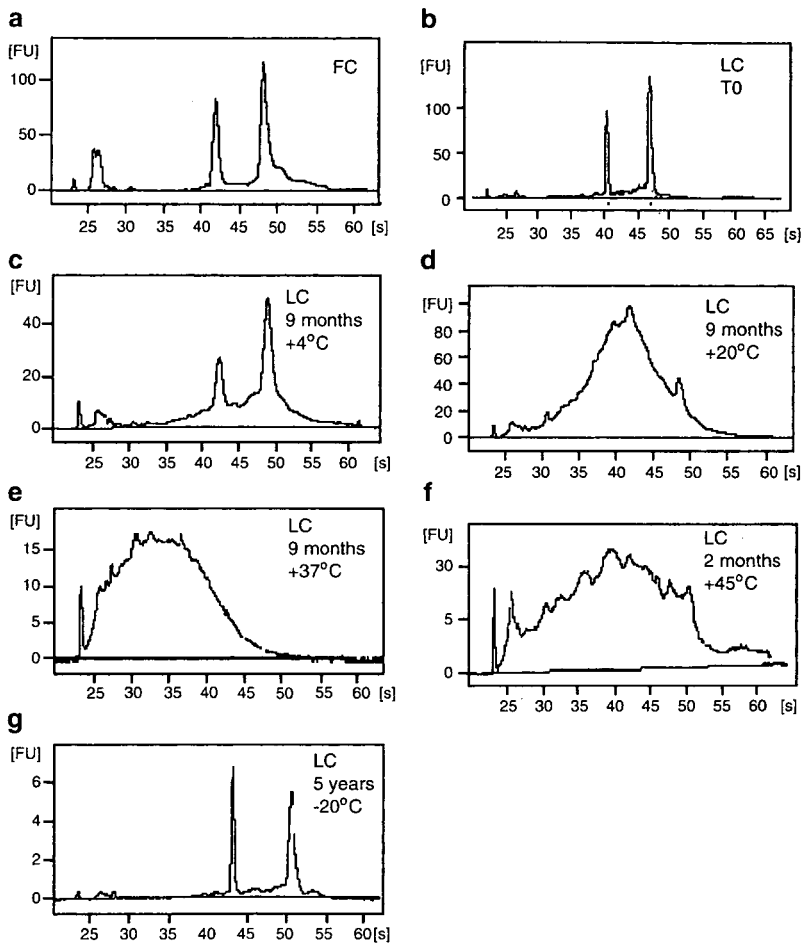

Figure 1 Determining the integrity of RNA with the Agilent 2100 Bioanalyzer. RNA 6000 Nano LabChip® were used. (**a**) Fresh K562 cells (FC); (**b**) lyophilized K562 cells (LC), $t=0$; (**c**) lyophilized K562 cells stored for 9 months at +4°C; (**d**) 9 months at +20°C and 9 months at +37°C (**e**); (**f**) lyophilized K562 cells incubated for 2 months at +45°C; (**g**) lyophilized K562 cells stored for 5 years at −20°C.

Table 1 Comparison of lyophilized and fresh K562 cells for fusion and control gene expression

	n	*ABL CN*	*GUS CN*	*BCR-ABL CN*
Fresh K562	7	4.7×10^4 (3.7×10^4–1.7×10^5)	1.2×10^5 (1.1×10^5–1.3×10^5)	6.0×10^4 (5.2×10^4–1.8×10^5)
Lyophilized K562 cells at t_0	7	1.7×10^5 (1.5×10^5–1.8×10^5)	1.3×10^5 (1.2×10^5–1.7×10^5)	2.4×10^5 (1.5×10^5–2.7×10^5)
Lyophilized K562 cells after 5 years at −20°C	7	2.3×10^5 (1.2×10^5–4.2×10^5)	2.3×10^5 (1.0×10^5–4.0×10^5)	2.1×10^5 (1.0×10^5–5.8×10^5)

Abbreviation: CN, copy number.
Results are median (95% range). Repeated experiments performed in one institution.
n = tested samples from different reverse transcription experiments.

Overtime stability of lyophilized cells

Analyses on stability of mRNA transcripts derived from lyophilized cells were performed in one laboratory (Marseille, France). Lyophilized K562 cells (batch 01/604) were stored from a few days to 12 months at various temperatures (Table 2) and for 5 years at −20°C. Storage at temperatures above 4°C resulted in degradation of RNA, which was not observed with material kept for 5 years at −20°C (Figure 1d–g). Nevertheless, RQ-PCR analysis revealed that *BCR-ABL* and control gene transcript levels were generally comparable to those detected in pre-storage samples despite exposure to a range of temperatures for prolonged periods (Figure 2).

International study – data received

The majority of laboratories returned the cycle threshold (C_t) values and CNs of standard curves and samples as requested. The data from the standard curves (with plasmids or cell lines) supplied by the participants were analyzed in order to compare the assay performance across laboratories. Data from laboratories 11, 15 and 20 were excluded since they did not return standard curve data for *ABL* and/or *BCR-ABL* amplifications, therefore the efficiencies of RQ-PCR could not be assessed. Nine laboratories performed four independent assays with four standard curves, while 10 laboratories used the same standard curve to determinate *ABL* and *BCR-ABL* CNs in the four vials. Ninety-two standard curves were analyzed (see Supplementary Table 1) for *ABL* ($n=46$) and *BCR-ABL* ($n=46$). Data generated by experiments with RQ-PCR efficiency lower than 90% ($n=4$ for *ABL*, $n=2$ for *BCR-ABL* and $n=1$ for both *ABL* and *BCR-ABL*) were excluded from further analysis. The remaining experiments ($n=42$ for *ABL* and $n=43$ for *BCR-ABL*) in this inter-laboratory setting showed a mean efficiency for *ABL* and *BCR-ABL* amplifications of $95\pm6\%$ and $94\pm4\%$, respectively. The average slopes for *ABL* and *BCR-ABL* amplifications were -3.45 ± 0.14 and -3.47 ± 0.12, respectively.

International study – BCR-ABL ΔC_t values and CNs

BCR-ABL C_t and CNs were calculated from the returned data from the analyses performed on the four neat vials and from the 1/10 dilution in water for each laboratory (see Supplementary Table 2 for crude values: A for CN and B for NCN *BCR-ABL/ABL*). Only data fulfilling our quality criteria in terms of slope, efficiency, reproducibility and RNA quality of the analyzed sample (*ABL* transcript CN >1000 as required by the EAC protocol[17]) were taken into account (data not shown). The distribution of the ΔC_t values and *BCR-ABL* CNs in neat and dilution 10^{-1} vials are shown in Figure 3. *BCR-ABL* ΔC_ts were calculated by subtracting the mean *BCR-ABL* C_t value of the neat vial from that of the 10^{-1} dilution (Figure 3a). Results reported by three laboratories (9, 13 and 21) for particular vials were quite discrepant from the rest of the data set, potentially indicating laboratory errors in executing the dilutions or in data reporting. The remaining results showed a mean difference of $3.2C_t+/-0.7C_t$ between the neat and the 10^{-1} dilution vials, which was very close to the ΔC_t value equivalent to a 1−log difference based upon the slope of the *BCR-ABL* standard curve (3.45). Analysis of copy numbers showed a *BCR-ABL* level of $3.7\times10^5\pm6.6\times10^5$ in the neat sample and $7.2\times10^4\pm1.5\times10^5$ in the 10^{-1} diluted sample (Figure 3b and Supplementary Figure 1). The observed ratio between the neat and 10^{-1} sample was 8.5 ± 4.2. The distribution of *BCR-ABL* ΔC_t results and CNs (on the log ratio scale) appears to give a Gaussian (normal) distribution. This distribution was even accentuated when the ratios *BCR ABL/ABL* from the participating laboratories were used (Supplementary Figure 1).

Table 2 Time schedule for accelerated degradation study for lyophilized K562 cells

Incubation time	*Incubation temperature*			
	+4°C	*+20°C*	*+37°C*	*+45°C*
7 days	—	—	2 vials	2 vials
21 days	—	—	—	2 vials
1 month	2 vials	2 vials	2 vials	2 vials
2 months	—	—	2 vials	2 vials
6 months	2 vials	2 vials	2 vials	—
9 months	2 vials	2 vials	2 vials	—
12 months	2 vials	2 vials	2 vials	2 vials

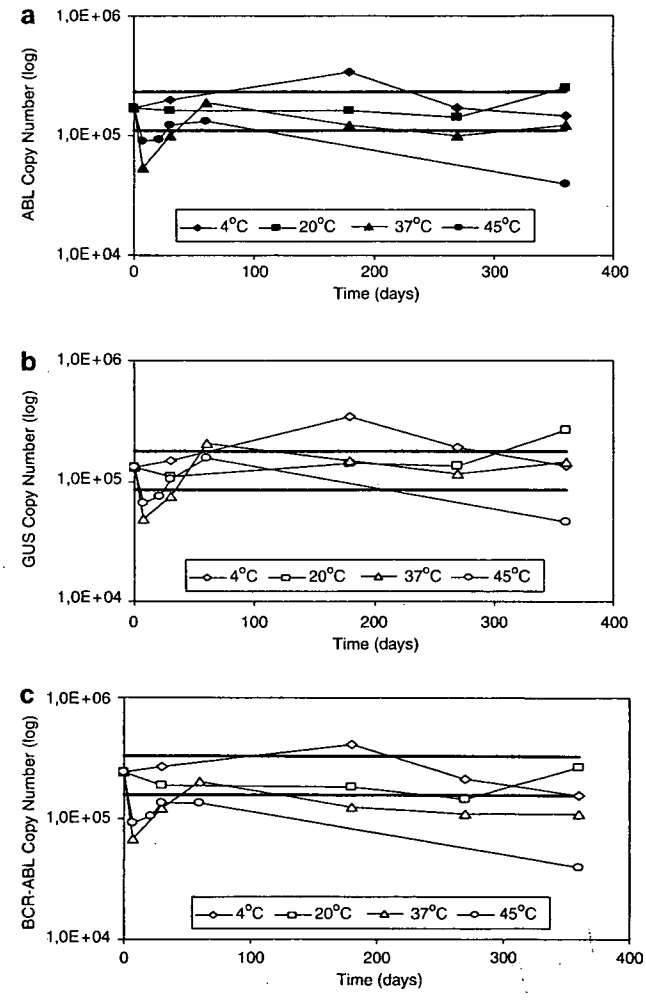

Figure 2 RQ-PCR analysis following accelerated degradation experiments. *ABL* (**a**), *GUS* (**b**) and *BCR-ABL* (**c**) copy numbers (CNs) in lyophilized K562 cells stored from zero to 360 days at +4°C, +20°C, +37°C or +45°C. For each condition, RNA was extracted by Trizol; RT and RQ-PCR were performed following the EAC protocol. Calibrated plasmid standards were used to construct standard curves allowing determination of CN for each transcript. Horizontal lines represent the 35% variation in CN compared to t_0 which are permitted by the French Association of Normalization (AFNOR), which is a legal agency for commercial products.

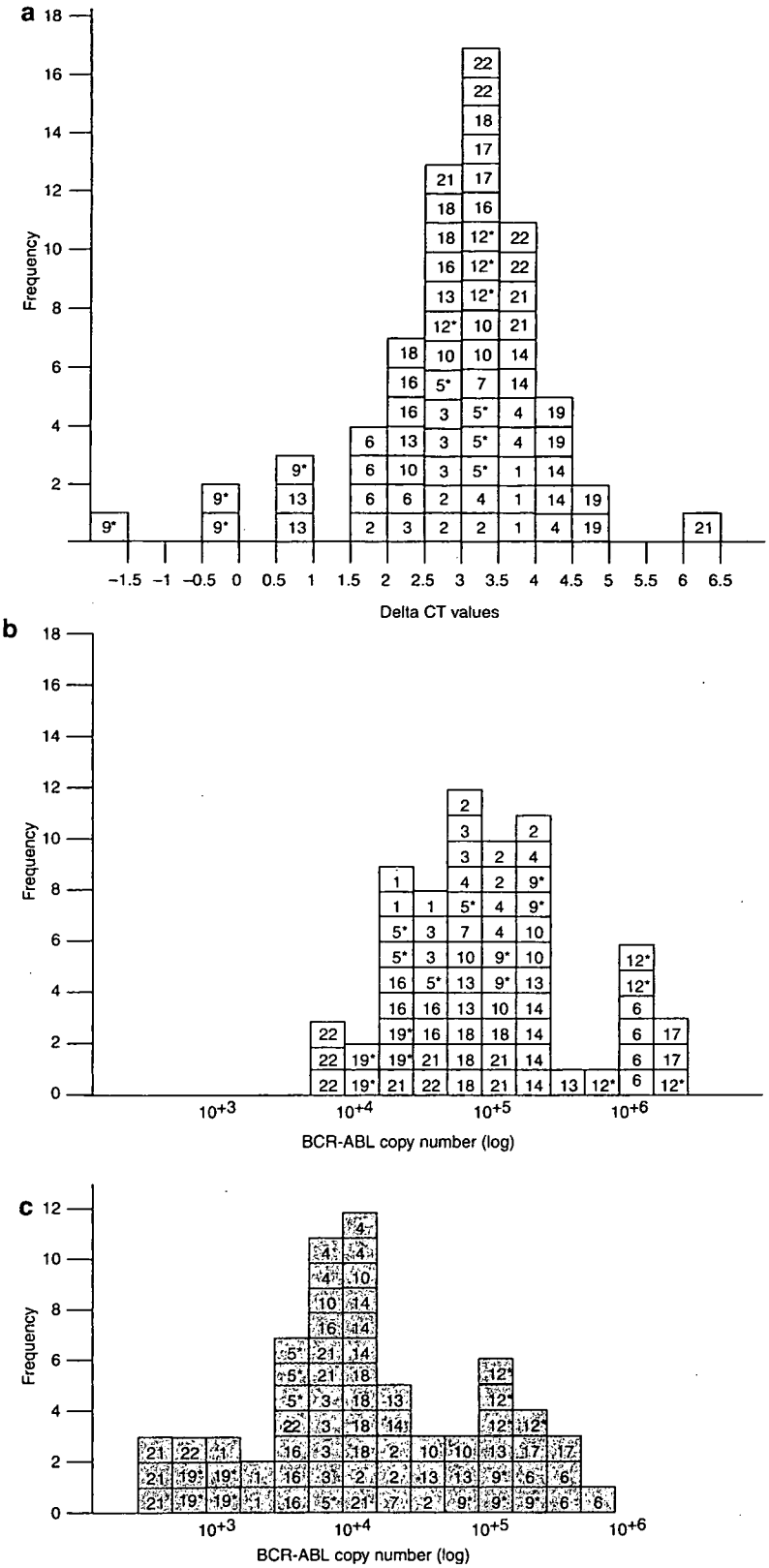

Figure 3 Reproducibility between 21 laboratories from four continents. Results with poor (as defined by EAC) or unknown efficiency of RQ-PCR were excluded. *Laboratories using calibrators other than EAC plasmids for their standard curves (Materials and methods). Each box is labelled with the laboratory code number. (**a**) *BCR-ABL* Delta C_t values between 1 in 10 dilution and undiluted lyophilized K562 cells. Each box represents the *BCR-ABL* ΔC_t value between the neat and 10e-1 dilution per vial. Each box represents the *BCR-ABL* copy number per vial from one laboratory for the neat sample (**b**) and 1:10 dilution in water (**c**). The *BCR-ABL* copy numbers are plotted on log scale.

Discussion

To serve as a biological standard, it is desirable that a preparation be stable for many years. From the Arrhenius model for accelerated degradation experiments used for proteins, the estimates of potency for samples stored at elevated temperature can be used to predict the long-term stability of proteins at various temperatures.[20,21] We applied this model to our RNA study and it was deduced that the material should be stable for at least 3 years at −20°C. Although it is clear that heating the biological material altered the integrity of the total cellular RNA, as determined by the Agilent bioanalyzer (Figure 1), the small mRNA transcripts detected by RQ-PCR remained relatively well preserved. Some variation in the relative level of *BCR-ABL* and the *GUS* control gene expression was observed between fresh and lyophilized K562 cells and in the latter following storage under different conditions. While it is possible that this reflects changes introduced in the lyophilization process and differential rates of transcript degradation,[22] inter-assay variability due to differences in the efficiency of RNA extraction, RT and PCR steps could also have made a significant contribution. Overall, these experiments clearly show that high levels of transcripts can be amplified from lyophilized cell preparations despite exposure to a wide range of ambient temperatures for prolonged periods of time.

The potential of lyophilized cell lines to provide relatively stable quality control reagents was further highlighted by the international study. Although some inter-laboratory variability was apparent, which was not unexpected and likely to reflect differences in experimental details, it is clear that shipment of lyophilized cell preparations at ambient temperature yielded RNA of sufficient quality to amplify high levels of *BCR-ABL* transcripts, with CN values comparable to those detected in primary patient samples.[18] This establishes a proof of principle, providing the first step towards the development of standard reagents based upon cell line dilutions that simulate clinically relevant levels of MRD. In CML, accurate detection and quantitation of *BCR-ABL* and control gene transcripts is fundamental to rapid identification of patients failing imatinib therapy due to resistance, who could benefit from treatment modification, in addition to directing the need for therapeutic intervention following allogeneic transplantation.[23–25] CML has provided a paradigm for molecular diagnostics and MRD monitoring, which is increasingly being applied to other subsets of leukemia to achieve a more rational treatment approach. The development of internationally recognized reference materials and external quality control rounds will be critical to ensure the reliable use of molecular diagnostics and monitoring of MRD for the appropriate management and clinical benefit of leukemia patients.

Acknowledgements

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

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Case Report

Successful Nonmyeloablative Bone Marrow Transplantation for Leukocyte Adhesion Deficiency Type I from an Unrelated Donor

Masahiro Tokunaga,[a] Koichi Miyamura,[a] Haruhiko Ohashi,[b] Naruhiko Ishiwada,[c] Seitaro Terakura,[d] Miyoko Ikeguchi,[a] Yachiyo Kuwatsuka,[a] Yoshihiro Inamoto,[a] Taku Oba,[a] Shigeru Tsuchiya,[e] Yoshihisa Kodera[a]

[a]*Department of Hematology, Japanese Red Cross Nagoya First Hospital, Nagoya, Japan;* [b]*Clinical Research Center for Blood Diseases, National Hospital Organization Nagoya Medical Center, Nagoya, Japan;* [c]*Department of Pediatrics, Chiba University Graduate School of Medicine, Chiba, Japan;* [d]*Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, Nagoya, Japan;* [e]*Department of Pediatrics, Tohoku University Graduate School of Medicine, Sendai, Japan*

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Abstract

Leukocyte adhesion deficiency type I (LAD-I) is an inherited immunodeficiency disorder caused by defective expression of the leukocyte integrins, namely, lymphocyte function-associated antigen 1, Mac-1, and p150,95, and is associated with obstructed cell adhesion, migration, and phagocytosis. Patients suffer from various bacterial or fungal infections and their prognoses are poor. The only curative treatment is hematopoietic stem cell transplantation. Conventional myeloablative transplantations have been performed, but with unsatisfactory results. We performed the first successful nonmyeloablative unrelated marrow transplantation for a 20-year-old female LAD-I patient, who suffered from recurrent and occasionally life-threatening infections such as cellulitis, gingivostomatitis, and sepsis. We adopted a preparative regimen with fludarabine, cyclophosphamide, and low-dose total-body irradiation, and tacrolimus and short-term methotrexate as immunosuppressants. This procedure was sufficiently immunosuppressive to obtain stable engraftment without remarkable complications, and graft-versus-host disease was controllable. Dramatic improvement of her disease was observed, supported by the normal expressions of integrins. Twenty one months after transplantation, she is well with a Karnofsky score of 100. Thus, nonmyeloablative transplantation is considered a feasible method for LAD-I.

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Key words: Leukocyte adhesion deficiency type I; Nonmyeloablative unrelated marrow transplantation

1. Introduction

Leukocyte adhesion deficiency type I (LAD-I) is a rare autosomal recessive disorder, caused by CD18-encoding gene mutations [1,2]. Since its first recognition in 1974, more than 200 patients have been reported all over the world, with less than 10 in Japan. β_2 integrins, that is, lymphocyte function-associated antigen 1 (LFA-1, CD11a/CD18), Mac-1 (CD11b/CD18), and p150,95 (CD11c/CD18), are composed of unique α and common β subunits (CD18), and, in patients with LAD-I, cannot be expressed on the surface of leukocytes because of diminished or structurally abnormal CD18. As they are involved in adhesion, migration, and phagocytosis, the leukocytes cannot transmigrate from the bloodstream to the site of infection and act against microorganisms. Soon after birth, delayed umbilical cord detachment and leukocytosis are seen, and patients usually suffer from various bacterial or fungal infections. Their prognoses are poor, especially in the severe type [1]. The only curative treatment is allogeneic hematopoietic stem cell transplantation, and successful cases from both related [3,4] and unrelated [5,6] bone marrow donors, and even from related cord blood [7], have been reported. They all received myeloablative conditioning

Correspondence and reprint requests: Koichi Miyamura, Department of Hematology, Japanese Red Cross Nagoya First Hospital, 3-35, Michishita-cho, Nakamura-ku, Nagoya 453-8511, Japan; 81-52-481-5111; fax: 81-52-482-7733 (e-mail: miyamu@nagoya-1st.jrc.or.jp).

regimens, which most commonly included busulfan, cyclophosphamide, and etoposide, as they were naïve to chemotherapy and were thought to be at high risk for graft rejection. But these regimens did not always ensure stable engraftment [3], and life-threatening complications such as sepsis were sometimes observed before hematological recovery [5]. Nonmyeloablative hematopoietic stem cell transplantation (NST) and reduced-intensity hematopoietic stem cell transplantation (RIST) have been increasingly used for patients with malignancies [8-11]. Because of the lower toxicity, they are applied to patients with nonmalignant diseases for whom transplant-related mortality with conventional myeloablative regimens is unacceptable [11-14]. We and many other investigators have had successful experiences with NST or RIST for patients with immunodeficiencies, while avoiding severe posttransplantation infections [13-15]. Here, we report a nonmyeloablative unrelated marrow transplantation for a patient with LAD-I, who achieved stable engraftment and survival longer than 21 months.

2. Case Report

A 20-year-old woman was admitted to our hospital for bone marrow transplantation (BMT) for LAD-I. She was the second child of a non-consanguineous Japanese couple, and had been hospitalized at 1 week of age because of perirectal abscess and delayed umbilical cord separation. Since then, she had suffered from various infections such as omphalitis, bronchitis, pyelitis, and cellulitis. Based on evidence of defective expression of LFA-1 and Mac-1 on her leukocytes, she was diagnosed with the severe form of LAD-I when she was 10 months old. Although she did fairly well in early childhood, she had to be administered intravenously with antibiotics repeatedly since adolescence for impaired wound healing and recurrent cellulitis in her limbs. She also underwent life-threatening infections such as sepsis and severe gingivostomatitis that made her unable to eat. Her quality of life became severely deteriorative. With no suitable related donor available, she decided to receive unrelated BMT and gave us written informed consent.

On admission, she was of short stature but with a relatively good nutritional status. Her oral mucosa was erosive and her gingivae were reddish and atrophic. Her tongue was ulcerous and covered with fur. A left submandibular lymph node was palpable and accompanied by tenderness. A few lesions with rubor were seen over her limbs.

Her white blood cell count was 11.2×10^9/L. Biochemical testing showed elevation of serum amylase derived from salivary glands, and roughly normal liver and renal functions. Serologic testing demonstrated polyclonal gammopathy (IgG 24.26 g/L; IgA 8.68 g/L; IgM 1.91 g/L) and slight elevation of C-reactive protein (36 mg/L). On flow cytometric evaluation, less than 1% of her peripheral blood granulocytes expressed CD18 (Figure 1A). Bacteriocidal activity of neutrophils was normal, but natural killer cell activity, which was mediated by LFA-1, was decreased. From her oral mucosa, multidrug-resistant *Pseudomonas aeruginosa* was isolated. She had mild splenomegaly and periaortic lymphadenopathies demonstrated by computed tomography but no other inflammatory lesion in her abdominal and pleural cavities. On gallium whole-body scan, abnormal uptake, implying an inflammatory focus, was seen at her left parotid and submandibular glands.

We used a nonmyeloablative regimen with fludarabine 30 mg/m^2 on days –6 to –2 and cyclophosphamide 60 mg/kg on days –3 and –2, followed by 2 Gy of total-body irradiation on day –1. Graft-versus-host disease (GVHD) prophylaxis consisted of short-term methotrexate (10 mg/m^2 on day 1; 7 mg/m^2 on days 3 and 6) and continuous infusion of tacrolimus that started at an initial dose of 0.02 mg/kg/day 2 days before transplantation. Bone marrow cells from a sex-mismatched volunteer donor through the Japan Marrow Donor Program were infused without processing on day 0. They contained 2.86×10^8 nucleated cells/kilogram of recipient weight and 2.57×10^6 CD34$^+$ cells/kilogram. The graft was genetically matched at human leukocyte antigen (HLA)-A, B, and DRB1 loci in the graft-versus-host direction, and serologically mismatched at one B locus in the host-versus-graft direction. ABO blood type was matched, and the serologic status for cytomegalovirus was positive. To prevent infections before hematological recovery, oral ciprofloxacin and fluconazole were given. Granulocyte colony–stimulating factor was administered from day 7.

To assess donor chimerism from peripheral blood samples, we used 2 techniques: polymerase chain reaction–based analyses of polymorphic short tandem repeat (STR) regions [16], and fluorescent in situ hybridization (FISH) for the sex chromosomes.

The clinical course of this patient is shown in Figure 2. No remarkable regimen-related toxicity was observed except mild self-limiting cystitis. Hematological recovery was as follows: the neutrophil count exceeded 0.5×10^9/L 15 days after transplantation, and the platelet count 50×10^9/L on day 25, with the final platelet transfusion on day 13. The duration of neutropenia (neutrophils less than 0.5×10^9/L) was 11 days, and intravenous antibiotics were needed for the treatment of gingivostomatitis. However, she did not develop any other significant infection. Soon after neutrophilic recovery, her gingivostomatitis improved dramatically and disappeared for the first time in 10 years. Swelling of the submandibular lymph node also disappeared. No recurrence of cellulitis was seen, and normalization of C-reactive protein was achieved on day 39. During the peritransplantation period, she developed no viral infection including cytomegalovirus antigenemia.

On day 28, degrees of donor chimerism assessed by the STR analysis were: T-cells 97.1%, and granulocytes 84.1%, indicating an initial donor cell engraftment. A decrease in donor content (granulocytes: about 50%), assessed by FISH using the sample on day 42, made us commence tapering tacrolimus in the hope of inducing a graft-versus-marrow effect to eliminate residual host cell production and prevent graft rejection. On day 56, donor chimerism assessed by STR analysis recovered (T-cells 100%; granulocytes 82.5%), followed by complete donor chimerism among both lineages on day 109. Donor lymphocyte infusion to promote the engraftment was unnecessary.

Flow cytometric analyses of peripheral blood samples demonstrated that CD18 expression of granulocytes was 76.1% on day 56 and 100% on day 102 (Figure 1B and C), which correlated with the donor chimerism.

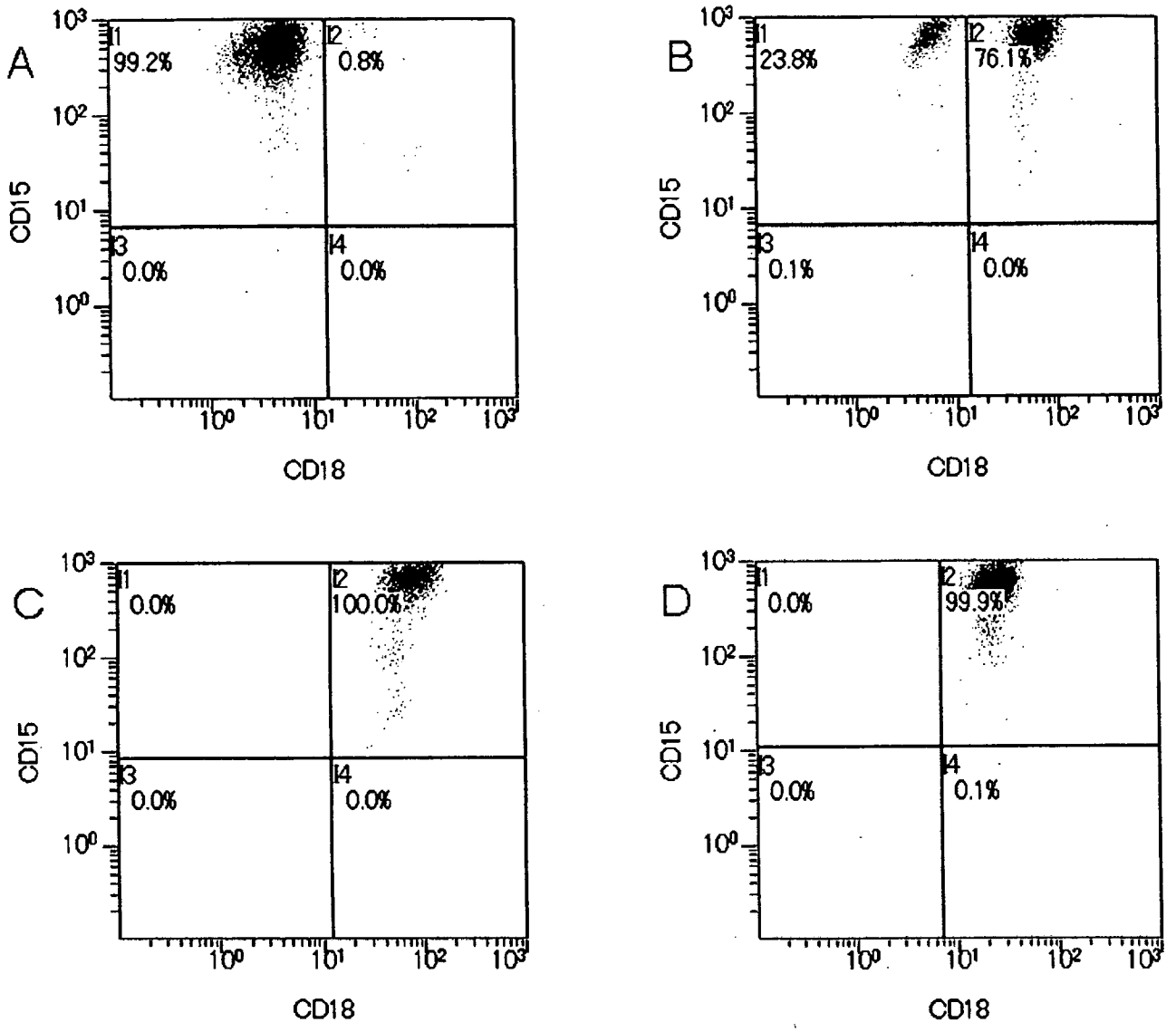

Figure 1. Improved expression of CD18 on granulocytes. Granulocytes from the peripheral blood samples were gated according to CD45 expression and side-scatter ($CD45^+$, high side-scatter) with a flow cytometer. All expressed CD15, a surface marker of granulocytes. These cells were analyzed regarding the expression of CD18. Less than 1% before transplantation (A), 76.1% on day 56 (B), and all cells on day 102 (C), expressed this molecule. D shows a healthy control.

On day 50, she developed skin eruptions over her face and neck, followed by elevation of liver enzymes from day 64, and oral mucosal lesions from day 75. She was diagnosed with chronic GVHD by a biopsy specimen from her lip. The skin and oral changes were mild and improved without systemic steroid administration. Her values of aspartate aminotransferase and alanine aminotransferase rose to 156 and 282 IU/L, respectively, but gradually and spontaneously normalized by 4 months from onset.

As of October, 2006, 21 months after transplantation, she was doing well in the outpatient clinic without any infection. Full donor chimerism was demonstrated at 18 months by FISH. With a Karnofsky score of 100, she has neither transplant-related sequela nor active GVHD. Although suppressed menstruation was observed after the preparative regimen, it was transient and her regular menstruation came back around day 100. Her gynecological hormone levels are normal.

3. Discussion

LAD-I is caused by CD18-encoding gene mutations such as missense, nonsense, splice defect, deletion, and insertion [2]. This case is a compound heterozygote with 2 different mutant alleles: one allele of maternal origin, with a missense mutation (C533T) resulting in a single amino acid substitution

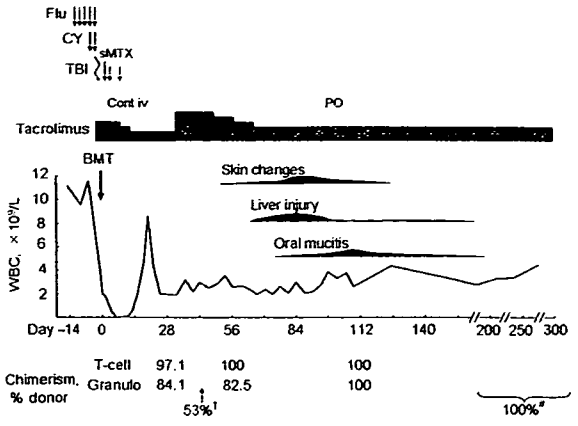

Figure 2. Clinical course of nonmyeloablative bone marrow transplantation. Donor chimerism on day 42 was assessed by examining morphologically segmented leukocytes by fluorescent in situ hybridization (FISH) (†). Full donor chimerism was observed at 6, 12, and 18 months by FISH for whole leukocytes (#). Flu indicates fludarabine; CY, cyclophosphamide; TBI, total-body irradiation; sMTX, short-term methotrexate; Cont iv, continuous intravenous; PO, per os; WBC, white blood cells; and Granulo, granulocytes.

(Pro178Leu), and the other from her father causing a defective expression of CD18 mRNA [17]. The reason for the latter is unknown but may be an abnormality in a CD18 promoter region.

With an extremely poor prognosis because of the recurrent life-threatening infections and the appearance of the multidrug-resistant *Pseudomonas aeruginosa*, she needed functional correction of this disease. Hematopoietic cell transplantation is now the only method until gene therapy becomes of practical use.

To our knowledge, this is the first successful clinical case of NST for LAD-I, although Sokolic et al succeeded in allogeneic BMT in canine LAD dogs after a nonmyeloablative regimen with busulfan only [18]. Two human LAD-I patients receiving unrelated BMT with reduced-intensity conditioning were reported recently, but neither survived longer than 16 months because of transplant-related complications [19,20].

LFA-1 is associated with T-cell cytotoxic activity, so it is natural that its absence on recipient cells should lower the risk of graft rejection. Indeed, a mouse anti-LFA-1 monoclonal antibody was once tried to facilitate engraftment for patients with other immunodeficiencies receiving T-cell–depleted (TCD) transplantation [21]. However, Thomas et al observed rejection in 4 of 9 LAD-I patients after HLA-nonidentical TCD transplantation, and even in 1 of 5 receiving T-cell replete grafts from HLA-matched related donors [3]. This may have been because of the insufficient bioavailability of busulfan, or because recipients had undergone no prior chemotherapy and their immune and marrow functions were robust. A fludarabine-cyclophosphamide regimen was reportedly sufficiently immunosuppressive to facilitate stable donor engraftment [9], and, by adding low-dose total-body irradiation to this regimen, we could overcome graft rejection despite the unrelated marrow and the relatively low number of $CD34^+$ cells infused.

On the other hand, it remains unclear whether the absence of LFA-1 exerts an influence on the development of GVHD, and the incidence and severity of GVHD among LAD-I patients have been a matter of controversy [3-6]. In this case, we used tacrolimus, and this is a stronger immunosuppressant than cyclosporine and may have served to prevent graft rejection and simultaneously control her GVHD.

We performed NST to avoid severe infections, especially by multidrug-resistant *Pseudomonas aeruginosa*. A nonmyeloablative regimen, in addition to its decreased toxicities, which may be favorable for patients with organ dysfunctions due to recurrent inflammations and antibiotics, has the advantages of mild mucitis and thus fewer opportunities for microorganisms to invade such lesions. Even in the case of rejection, recovery of the host cells can be expected. Accordingly, the risk of life-threatening infections must be lower compared with myeloablative regimens. Furthermore, in our case, transplanted bone marrow cells contained live, mature neutrophils, which may have transiently acted against microorganisms.

Even during the period around day 42, when the donor granulocyte chimerism transiently fell to about 50%, no recurrence of infections was observed. This is consistent with the previously reported fact that sustained engraftment with >20% donor neutrophils was sufficient to reverse the disease phenotype [3,5].

In conclusion, NST using fludarabine and cyclophosphamide can be considered a feasible treatment for LAD-I, and to evaluate the benefit of this procedure, further experience should be accumulated.

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Hyperglycemia During the Neutropenic Period Is Associated With a Poor Outcome in Patients Undergoing Myeloablative Allogeneic Hematopoietic Stem Cell Transplantation

Shigeo Fuji,[1] Sung-Won Kim,[1] Shin-ichiro Mori,[1] Takahiro Fukuda,[1] Shigemi Kamiya,[2] Satoshi Yamasaki,[1] Yuriko Morita-Hoshi,[1] Fusako Ohara-Waki,[1] Osamu Honda,[3] Setsuko Kuwahara,[2] Ryuji Tanosaki,[1] Yuji Heike,[1] Kensei Tobinai,[1] and Yoichi Takaue[1,4]

Background. Recipients of allogeneic hematopoietic stem cell transplantation (HSCT) frequently require support with parenteral nutrition and immunosuppressive drugs, which introduce the risk of hyperglycemia. Van den Berghe et al. showed that the strict glucose control improved the outcome of patients treated in the intensive care unit, and this point was evaluated in this study in a HSCT setting.

Methods. A cohort of 112 consecutive adult patients treated by myeloablative allogeneic HSCT between January 2002 and June 2006 was reviewed retrospectively. Twenty-one patients were excluded due to graft failure, preexisting infectious diseases, preexisting neutropenia or previous allogeneic HSCT. The remaining 91 patients were categorized according to mean fasting blood glucose (BG) level in the neutropenic period after conditioning: normoglycemia (BG <110 mg/dL, n=28), mild hyperglycemia (110 to 150 mg/dL, n=49), and moderate/severe (>150 mg/dL, n=14). The primary endpoint was the occurrence of febrile neutropenia (FN) and documented infection during neutropenia, and the secondary endpoints included organ dysfunction according to the definition used by van den Berghe, acute graft-versus-host disease (GVHD), overall survival, and nonrelapse mortality (NRM).

Results. Although the incidence of FN or documented infections was similar between the three groups, hyperglycemia was significantly associated with an increased risk of organ dysfunction, grade II–IV acute GVHD, and NRM.

Conclusions. While the results suggested an association between the degree of hyperglycemia during neutropenia and an increased risk of posttransplant complications and NRM, the possibility that intensive glucose control improves the outcome after HSCT can only be confirmed in a prospective randomized trial.

Keywords: Allogeneic transplantation, Hyperglycemia, Nonrelapse mortality, Acute graft-versus-host disease.

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Van den Berghe et al. showed with patients nursed in the intensive care unit (ICU) that the rigid control of hyperglycemia with intensive insulin therapy to keep the blood glucose level at 80–110 mg/dL reduced morbidity, including infections, and mortality compared to patients who received standard care maneuvers that maintained the level at <200 mg/dL (*1–3*). Although these results have been confirmed in several subsequent studies (*4–7*), the precise mechanism that underlies this association is unclear. In animal models, it has been shown that insulin itself has a direct inhibitory effect on the inflammation process (*8, 9*). However in human studies, it has been suggested that these benefits could be directly attributed to intense glucose control rather than to any pharmacological activity of administered insulin per se (*3, 4*).

Recipients of allogeneic hematopoietic stem cell transplantation (HSCT) suffer from serious complications including infection, graft-versus-host disease (GVHD) and organ dysfunction. They are also at higher risk of hyperglycemia due to the use of steroids for the treatment of graft-versus-host disease (GVHD), prolonged total parenteral nutrition (TPN), immunosuppressive drugs, and infectious complications (*10, 11*). This makes them susceptible to numerous serious complications, including multiple organ failure (*12–14*). In this study, we evaluated whether hyperglycemia during the cytopenic pe-

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[1] Department of Hematology and Stem Cell Transplantation, National Cancer Center Hospital, Tokyo, Japan.

[2] Division of Nutritional Management, National Cancer Center Hospital, Tokyo, Japan.

[3] Tokyo Anesthesiology Group, Tokyo, Japan.

[4] Address correspondence to: Yoichi Takaue, M.D., Department of Hematology, National Cancer Center Hospital., 5-1-1, Tsukiji, Chuo-Ku, Tokyo 104-0045, Japan.

E-mail: ytakaue@ncc.go.jp

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riod after conditioning for HSCT could be a significant risk factor for the subsequent clinical course.

PATIENTS AND METHODS

Patient Characteristics

A cohort of 112 consecutive adult patients who received myeloablative allogeneic HSCT between January 2002 and June 2006 at the National Cancer Center Hospital (Tokyo, Japan) was reviewed retrospectively. Twenty-one patients were excluded due to graft failure, pre-existing infectious diseases or neutropenia before HSCT, and previous allogeneic HSCT. The remaining 91 patients were subjected to further analysis, and their characteristics are listed in Table 1. Their median age was 36 years (range, 18–57 years), and their diagnosis included acute myeloid leukemia (AML, n=41), acute lymphoblastic leukemia (ALL, n=21), non-Hodgkin lymphoma (NHL, n=13), myelodysplastic syndrome (MDS, n=10), and chronic myelogenous leukemia (n=6). Standard-risk patients included those with acute leukemia in first complete remission, chronic leukemia in first chronic phase, MDS in refractory anemia, and NHL in complete remission, and the remaining patients were categorized as high-risk. Forty-six and 45 patients received a graft from a related donor and an unrelated donor, respectively. Stem cell sources included bone marrow (n=46), peripheral blood (n=41), and cord blood cells (n=4). In this study, only two patients were diagnosed as type 2 diabetes mellitus before HSCT, which reflects the low prevalence of this condition in Japan, especially in younger patients who can be the target of allogeneic HSCT with a myeloablative conditioning regimen. These two diabetic patients were included in the moderate and severe hyperglycemia group. None of the patients, including these two patients, had major organ dysfunction or diabetic complications before HSCT. For the transplantation procedure, signed informed consent was obtained according to the Declaration of Helsinki.

Transplantation Procedures

All patients received a myeloablative conditioning regimen that included oral busulfan (BU) plus cyclophosphamide (CY, n=45), CY plus 12 Gy total body irradiation (TBI, n=43) or cytarabine (CA) plus CY plus TBI (n=3: Table 1). GVHD prophylaxis included cyclosporine- (n=62) and tacrolimus-based regimens (n=29), with an additional short course of methotrexate (MTX) in 89 patients. Granulocyte

TABLE 1. Patient characteristics

Variable	Normoglycemia (<110 mg/dl)	Mild hyperglycemia (110–150 mg/dl)	Moderate and severe hyperglycemia (>150 mg/dl)
N	28	49	14
Blood glucose, median mg/dl (range)	104 (81–109)	120 (110–150)	168 (150–211)
Age, median years (range)	31 (21–52)	36 (18–57)	45 (30–57)
<40	20 (71)	32 (65)	4 (29)
≥40	8 (29)	17 (35)	10 (71)
Sex			
Male	9 (32)	34 (69)	8 (57)
Female	19 (68)	15 (31)	6 (43)
Disease risk			
Standard	16 (57)	18 (37)	6 (43)
High	12 (43)	31 (63)	8 (57)
Conditioning			
TBI-containing	11 (39)	26 (53)	9 (64)
Non-TBI-containing	17 (61)	23 (47)	5 (36)
GVHD prophylaxis			
Cyclosporine-based	24 (86)	33 (67)	5 (36)
Tacrolimus-based	4 (14)	16 (33)	9 (74)
Relation to donor			
Related	19 (68)	24 (49)	3 (21)
Unrelated	9 (32)	25 (51)	11 (79)
Stem cell source			
Bone marrow	11 (39)	24 (49)	11 (79)
PBSC	16 (57)	22 (45)	3 (21)
Cord blood	1 (4)	3 (6)	0 (0)
HLA match			
Match	25 (89)	34 (69)	10 (71)
Mismatch	3 (11)	15 (31)	4 (29)

Data are n (%) unless noted.
TBI, total body irradiation; GVHD, graft-versus-host disease; PBSC, peripheral blood stem cells; HLA, human leukocyte antigen.

colony-stimulating factor (G-CSF) was administered in all patients from day +6 after transplantation until engraftment. Most patients received ciprofloxacin (200 mg orally three times daily) for bacterial prophylaxis until neutrophil engraftment. Fluconazole (100 mg once daily) was administered for fungal prophylaxis. Low-dose acyclovir was given for prophylaxis against herpes simplex virus and varicella zoster virus until the cessation of immunosuppressive agents. Prophylaxis against *Pneumocystis jiroveci* infection consisted of trimethoprim-sulfamethoxazole (400 mg of sulfamethoxazole once daily) from the first day of conditioning to day −3 of transplantation, and from day +28 until day +180 or the cessation of immunosuppressive agents. Patients who developed fever during the neutropenic period were treated with cefepime, and additional agents including vancomycin, aminoglycosides and amphotericin B were given as clinically indicated. Neutrophil engraftment was defined as the first of 3 consecutive days after transplantation that the absolute neutrophil count exceeded 0.5×10^9/L.

Grouping of Patients

Patients were categorized according to the mean blood glucose (BG) level in the preengraftment neutropenic period: normoglycemia BG maintained at <110 mg/dL (group 1, n=28), mild hyperglycemia at 110–150 mg/dL (group 2, n=49), and moderate/severe hyperglycemia at >150 mg/dL (group 3, n=14). Blood glucose level was routinely tested in the morning at least three times a week. Daily caloric intake was calculated by dietitian following the chart record.

Outcome Measures

The primary outcome measure was the occurrence of febrile neutropenia (FN) and documented infection including bacteremia, pneumonia and central venous catheter infection in the neutropenic period. Secondary outcome measurements were organ dysfunction in the neutropenic period, acute GVHD, overall survival (OS) and nonrelapse mortality (NRM). Organ dysfunction was defined with reference to van den Berghe (*5–7*) as follows: 1) hypercreatininemia: serum creatinine level ≥2.0 mg/dL or more than twice the baseline; 2) hyperbilirubinemia: serum total bilirubin level ≥2.0 mg/dL; and 3) increased inflammatory markers: serum C-reactive protein (CRP) level ≥15 mg/dL. Acute GVHD was graded by the Consensus Criteria (*15*).

Statistical Analyses

Standard descriptive statistics were used. The Student's *t*-test, chi-square, and Wilcoxon rank-sum tests were used to compare clinical and patient characteristics. Multiple logistic regression analysis was conducted to ascertain odds ratios (ORs) and 95% confidence intervals (CIs). OS was estimated using Kaplan-Meier curves. The cumulative incidences of NRM were estimated based on a Cox regression model for the cause-specific hazards by treating progressive disease or relapse as a competing event. Cox proportional hazard models were used for multivariate analysis of variables on NRM and OS after HCT. Clinical factors that were assessed for their association with NRM and OS included patient age, sex, conditioning regimen (TBI-based vs. non-TBI-based), donor [human leukocyte antigen (HLA)-matched vs. HLA-mismatched, related vs. unrelated], GVHD prophylaxis (cyclosporine-based vs. tacrolimus-based) and disease risk (standard vs. high). Factors with $P<0.10$ in the univariate analyses were subjected to a multivariate analysis. A level of $P<0.05$ was defined as statistically significant. All P values are two-sided. All analyses were performed using SPSS 10.0 statistical software (Chicago, IL).

RESULTS

Patients and Transplantation Characteristics

The median ages of the patients in the normoglycemia, mild hyperglycemia, and moderate/severe hyperglycemia groups were, respectively, 31, 36, and 45 years. The percentages of patients who received graft from an unrelated donor were 32%, 51%, and 79%, and the percentages of patients who received GVHD prophylaxis with tacrolimus were 14%, 33%, and 74%. To clarify the risk factor to be included in moderate and severe hyperglycemia group, logistic analysis was performed, which showed older age and GVHD prophylaxis with tacrolimus were associated with moderate and severe hyperglycemia [$P=0.04$, OR 3.9 (1.1–14.0), and $P=0.01$, OR 5.5 (1.5–20.3), respectively], and there was a trend that patients who received stem cell from unrelated donor were associated with moderate and severe hyperglycemia [$P=0.07$, OR 3.6 (0.9–14.2)]. Multiple logistic analysis showed age more than 40 years old and GVHD prophylaxis with tacrolimus were associated with moderate and severe hyperglycemia [$P=0.042$, OR 4.1 (1.1–15.7), and $P=0.01$, OR 5.8 (1.5–22.1), respectively].

Although in practice we generally keep the parenteral glucose dose relatively low to avoid severe metabolic complications including hyperglycemia and hyperlipidemia during the acute phase of allogeneic HSCT, the possibility that the dose of parenteral nutrition affects the blood glucose level should be explored. We calculated the total caloric intake by combining both oral and parenteral nutrition. Although the mild hyperglycemia group received significantly more parenteral nutrition than the normoglycemia group (group 1 694+322 kcal/day vs. group 2 969+383 kcal/day), overall there was no essential difference in caloric intake between the three groups (1070+303 kcal/day, 1190+393 kcal/day, 1045+530 kcal/day, respectively). The median duration of the follow-up time in surviving patients was 809 days (range, 132-1530 days) in group 1, 369 days (105–1550 days) in group 2, and 587 days (170–774 days) in group 3. Described as hydrocortisone-equivalent dose, the median dose of corticosteroid used during neutropenia was 0 mg (0–1610 mg) in group 1, 100 mg (0–9700 mg) in group 2, and 375 mg (0–2468 mg) in group 3. Statistically more dose of corticosteroid was used in group 2 and group 3, compared with group 1.

Primary Endpoints

The incidence of FN and documented infections is summarized in Table 2. The incidences of FN and documented infections including bacteremia, pneumonia, and central venous catheter infection in groups 1, 2 and 3 were, respectively, 89% and 32% (25%, 4% and 11%), 88% and 20% (16%, 6% and 6%), and 98% and 43% (36%, 14% and 14%). Overall, no statistically significant difference was observed between the three groups in the incidence of infectious episodes, including FN and documented infections.

TABLE 2. Endpoints

Variable	Normoglycemia (<110 mg/dl)	Mild hyperglycemia (110–150 mg/dl)	Moderate and severe hyperglycemia (>150 mg/dl)
N	28	49	14
Febrile neutropenia	23 (89)	43 (88)	13 (98)
Documented infection	9 (32)	10 (20)	6 (43)
Bacteremia	7 (25)	8 (16)	5 (36)
Pneumonia	1 (4)	3 (6)	2 (14)
Central-venous catheter infection	3 (11)	3 (6)	2 (14)
Organ dysfunction			
Hypercreatininemia	1 (4)	4 (8)	4 (29)
Hyperbilirubinemia	3 (11)	11 (22)	6 (43)
Increased inflammatory markers	4 (14)	15 (31)	9 (64)

Data are n (%).
Hypercreatininemia, serum creatinine level ≥2.0 mg/dl or more than twice of baseline; hyperbilirubinemia, serum bilirubin level ≥2.0 mg/dl; increased inflammatory markers, serum C-reactive protein level ≥15 mg/dl.

Secondary Endpoints

The incidence of hypercreatininemia was 4% in group 1, 8% in group 2 and 29% in group 3, as summarized in Table 2, and that in group 3 was significantly higher than those in group 1 (OR 10.8, 95% CI 1.1–108.6; $P=0.018$) and group 2 (OR 4.5, 95% CI 1.0–21.1; $P=0.043$). The incidence of hyperbilirubinemia was, respectively, 11%, 22% and 43%, in the three groups, and that in group 3 was significantly higher than that in group 1 (OR 6.3, 95% CI 1.3–30.9; $P=0.017$). The incidence of increased inflammatory markers was, respectively, 14%, 31% and 64%, and that in group 3 was significantly higher than those in group 1 (OR 10.8, 95% CI 2.4–49.5; $P<0.001$) and group 2 (OR 4.1, 95% CI 1.2–14.3; $P=0.022$). Multiple logistic regression analysis showed that the degree of hyperglycemia was associated with hypercreatininemia, hyperbilirubinemia, and increased inflammatory markers (Table 3).

TABLE 3. Multiple logistic regression analysis for organ dysfunction and multiple variate analysis for acute GVHD, nonrelapse mortality, and overall survival

Outcomes and variables	Odds/hazard ratio	95% CI	*P* value
Multiple logistic regression analysis			
Hypercreatininemia			
Hyperglycemia	5.2	1.1–24.6	0.039
Hyperbilirubinemia			
Hyperglycemia	4.9	1.6–14.9	0.005
Increased inflammatory markers			
Hyperglycemia	6.7	2.2–20.3	0.001
Tacrolimus-based	6.9	1.6–30.5	0.011
Multivariate analysis (Cox-proportional hazard model)			
Acute GVHD			
Hyperglycemia	2.3	1.2–4.3	0.013
Disease risk (high)	2.3	1.0–5.1	0.047
HLA mismatch	2.8	1.3–5.9	0.009
Nonrelapse mortality			
Hyperglycemia	2.9	1.2–6.6	0.013
Disease risk (high)	2.7	0.9–8.7	0.091
Overall survival			
Hyperglycemia	2.0	1.1–3.6	0.019
TBI-containing	2.3	1.1–5.0	0.035
Disease risk (high)	1.9	0.9–4.1	0.10

Odds ratios are presented for multiple logistic regression analysis; hazard ratios are presented for multivariate analysis.
GVHD, graft versus host disease; TBI, total body irradiation.

The cumulative incidence of grade II–IV acute GVHD is shown in Figure 1. The degree of hyperglycemia was associated with a higher incidence of grade II–IV acute GVHD

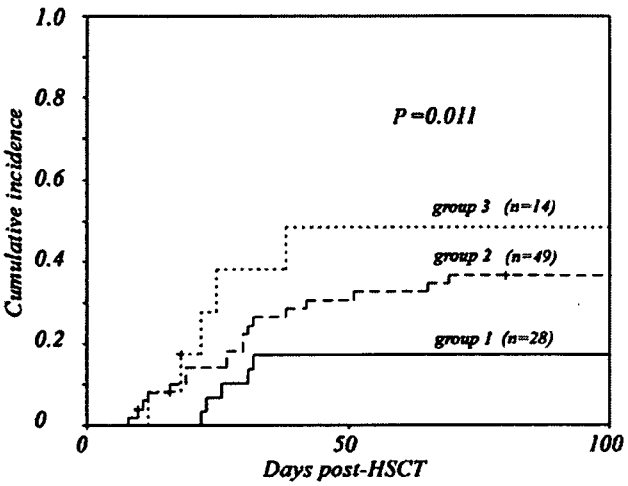

FIGURE 1. Cumulative incidence of acute GVHD grade II–IV stratified according to the mean glucose level during neutropenia. Group 1 included patients with normoglycemia, group 2 included patients with mild hyperglycemia, and group 3 included patients with moderate and severe hyperglycemia.

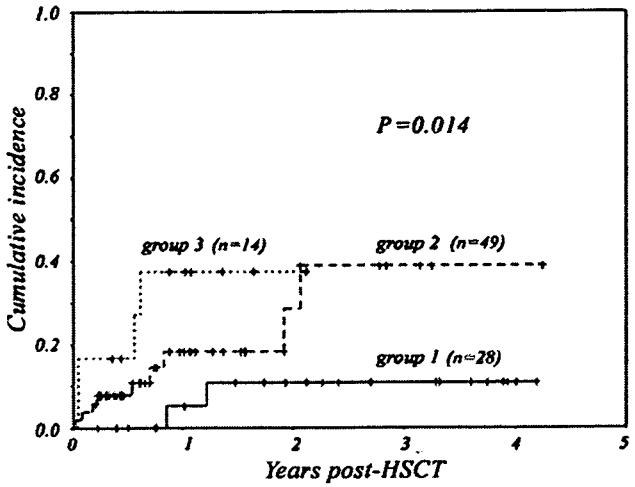

FIGURE 2. Cumulative incidence of treatment-related mortality stratified according to the mean glucose level during neutropenia.

(P=0.002). A Cox proportional hazard model showed that hyperglycemia, high-risk underlying disease, and HLA mismatch were risk factors for grade II-IV acute GVHD (Table 3).

The cumulative incidence of NRM was, respectively, 5%, 17%, and 35% at 1 year, and was significantly related to the degree of hyperglycemia (P=0.014; Fig. 2). The probability of OS was, respectively, 88%, 70%, and 56%, and was significantly associated with hyperglycemia (P=0.008; Fig. 3). A Cox proportional hazard model showed that the degree of hyperglycemia was associated with NRM and OS (Table 3).

DISCUSSION

In this study, we evaluated whether hyperglycemia during the cytopenic period after conditioning for HSCT could be a significant risk factor for the subsequent clinical course. Infectious diseases remain a major cause of morbidity and mortality in patients who receive HSCT, and we speculated that this might be exaggerated in the presence of hyperglycemia. Alternatively, hyperglycemia can be caused by infectious diseases and also aggravates infectious diseases to lead to a vicious cycle, with resultant morbidities that include organ dysfunction and mortality. Theoretically, strict glucose control should prevent this vicious cycle and help to reduce morbidity and mortality in patients after HSCT, as shown previously in ICU settings (*1*, *2*). However, in this study the incidences of FN and documented infections were not different among the three groups. On the other hand, we found that hyperglycemia was associated with organ dysfunction and increased inflammatory markers, which was consistent with previous reports that demonstrated the impact of hyperglycemia on clinical outcomes of patients suffering from nonhematological diseases (*1–3*, *12–14*). Additionally, a multivariate analysis showed that hyperglycemia was a risk factor for acute GVHD.

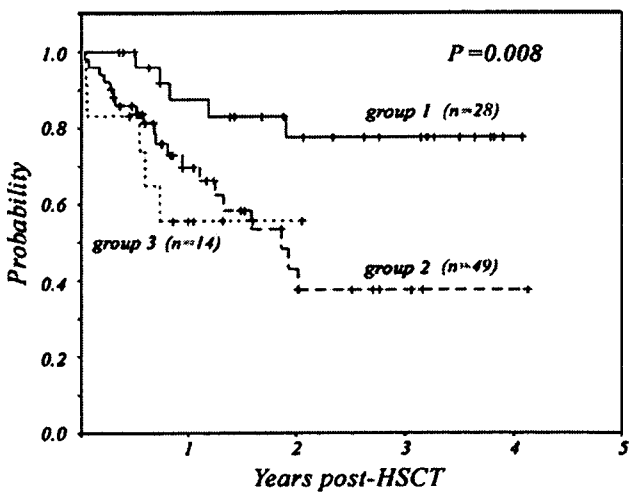

FIGURE 3. Overall survival stratified according to the mean glucose level during neutropenia.

The reason for the association between early hyperglycemia and late complications needs to be clarified. The increase in the levels of circulating cytokines due to hyperglycemia may further aggravate hyperglycemia itself (*16–21*). Therefore, this condition which occurs during the critical period of neutropenia before engraftment may influence the afferent phase of acute GVHD, as suggested by Ferrara et al. Elevated cytokine levels during the afferent phase then lead to subsequent acute GVHD in the effector phase (*22*, *23*). Teshima et al. reported that the effector phase of acute GVHD is not antigen-specific and inflammatory cytokines mediate target destruction (*24*), and other reports have shown that inflammatory cytokines were required in acute GVHD and these molecules can cause tissue damage (*25–27*). With these reports in mind, it is reasonable to speculate that the aggravated production of inflammatory cytokines by hyperglycemia may be a risk factor in the pathogenesis of acute GVHD and organ dysfunction.

This study has several limitations, including heterogeneous patient populations and a retrospective nature. First, hyperglycemia can be caused by infection itself and it has been previously shown that the level of hyperglycemia was correlated with the severity of illness (*4*). In this retrospective study, we could not confirm whether hyperglycemia directly influenced organ dysfunction or increased inflammatory markers. Furthermore, statistically more corticosteroid was used in the group of moderate and severe hyperglycemia, and statistically more parenteral nutrition was used in the group of mild hyperglycemia. However, the observation that hyperglycemia and the severity of illness were independently associated with a worse prognosis has been well confirmed in the ICU setting (*4*), and several prospective studies have shown that intensive glucose control reduced both morbidity and mortality (*1*, *2*). Considering these findings, we suggest that our data still support the possibility that the degree of hyperglycemia was associated with morbidity and mortality in the allogeneic HSCT setting. Second, we must consider that the patients who developed moderate and severe hyperglycemia included older patients, those who received more unrelated grafts, and those who received tacrolimus compared to other groups. In terms of immunosuppressive drugs, tacrolimus has recently become a preferred immunosuppressive drug for GVHD prophylaxis in unrelated or HLA-mismatched HSCT, based on the results of two Japanese studies, which showed that, compared to cyclosporine, tacrolimus was associated with a lower incidence of acute GVHD and better overall survival, which were similar to those in related HSCT, even

after HSCT with alternative donors, including unrelated donors (*28*, *29*). Therefore, the effect of unrelated graft and tacrolimus on the incidence of acute GVHD and NRM might not be significant in this study.

The effects of tacrolimus on hyperglycemia, hyperbilirubinemia, and hypercreatininemia need to be clarified. It is well known that hyperglycemia occurs more often in patients receiving tacrolimus than in those receiving cyclosporine (*30–32*). In the present study, patients receiving tacrolimus were more likely to have moderate to severe hyperglycemia. However, the association of hyperbilirubinemia with tacrolimus has not been previously reported and two other studies (*33*, *34*) showed that cyclosporine was more likely to cause hyperbilirubinemia than tacrolimus after allogeneic HSCT or kidney transplantation. Although the relative nephrotoxicity attributed to tacrolimus compared to cyclosporine has been controversial (*30*, *33*, *35*), studies that have reported such nephrotoxicity used a higher target tacrolimus level (>20 ng/ml) (*30*, *35*). On the other hand, it has been reported that the use of lower levels of tacrolimus (10–15 ng/ml in our hospital) was associated with reduced complications in allogeneic HSCT (*36*, *37*), with no difference in the incidence of hypercreatininemia compared to cyclosporine (*33*). Based on a consideration of all of these results, we think that tacrolimus might not be the direct cause of hypercreatininemia in this study. Finally, due to the nature of this retrospective study, during the period evaluated we did not apply any consistent protocol for glucose control and nutritional support, although we tried to avoid severe hyperglycemia (BG ≥200 mg/dl), which certainly biases the interpretation of the data, although it has been reported that the overall glucose level, rather than the dose of insulin administered, directly influenced the outcome of patients (*3*).

Even with these limitations, we believe that our observation is still of value in considering the clinical impact of the strict control of hyperglycemia during the early phase of HSCT. To confirm our preliminary observation, a prospective pilot study is underway to assess the effect of intensive glucose control after HSCT. If this pilot study shows a beneficial effect of intensive glucose control, a prospective randomized trial would be warranted to confirm the possibility that intensive glucose control improves the outcome after HSCT. Additionally, in this ongoing pilot study, we evaluate the diurnal blood glucose and insulin levels, including postprandial levels, to detect hyperglycemia more precisely before transplantation since the level of HgA1c is affected by both the blood glucose level and the turnover rate of red blood cells, and would not precisely correlate with the true mean blood glucose level in patients who received courses of blood transfusion for anemia.

In conclusion, the association of the degree of hyperglycemia during neutropenia and an increased risk of post-transplant complications and NRM was suggested, but the possibility that intensive glucose control improves the outcome after HSCT would only be confirmed in a prospective randomized trial.

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Unrelated-Donor Bone Marrow Transplantation with a Conditioning Regimen Including Fludarabine, Busulfan, and 4 Gy Total Body Irradiation

Yasushi Onishi, Shin-ichiro Mori, Shigeru Kusumoto, Kyoko Sugimoto, Daigo Akahane, Yuriko Morita-Hoshi, Sung-Won Kim, Takahiro Fukuda, Yuji Heike, Ryuji Tanosaki, Kensei Tobinai, Yoichi Takaue

Division of Hematology and Hematopoietic Stem Cell Transplantation, National Cancer Center Hospital, Tokyo, Japan

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Abstract

We investigated the feasibility of reduced-intensity conditioning with 4 Gy total body irradiation, fludarabine (30 mg/m^2 for 6 days), and busulfan (4 mg/kg for 2 days) for bone marrow transplantation from a serologically HLA-matched unrelated donor. Seventeen adult patients (median age, 55 years; range, 27-67 years) with various hematologic malignancies (6 in remission, 11 not in remission) were treated. Successful engraftment was achieved in all patients at a median of day 18 (range, day 14-35) after transplantation, although subsequent secondary graft failure was observed in 2 patients. The cumulative incidence of acute graft-versus-host disease (GVHD) of grades II to IV at day 100 was 48%. With a median follow-up of 286 days (range, 56-687 days), the rates of 1-year overall survival, 100-day nonrelapse mortality, and 1-year nonrelapse mortality were 41%, 14%, and 46%, respectively. Eleven patients died, and the causes of death were relapse (n = 4), pulmonary complications (n = 4), acute GVHD (n = 2), and sepsis (n = 1). The remaining 6 patients (at transplantation, 2 were in remission, and 4 were not in remission) are currently still in remission. These results suggest that this regimen reduces the risk of graft failure, but further studies are needed to ameliorate transplantation-related toxicities, primarily GVHD and/or pulmonary complications.

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Key words: Unrelated donor bone marrow transplantation; Fludarabine; Busulfan, TBI

1. Introduction

Although allogeneic hematopoietic stem cell transplantation (HSCT) is a possible curative approach for patients with various hematologic malignancies, only 30% to 40% of patients in Japan have an appropriate family donor available [1]. Hence, the application of unrelated-donor transplantation using bone marrow or cord blood cells has been expanding. Another area of current interest is the application of reduced-intensity conditioning regimens, mostly incorporating fludarabine as a primary agent, because conventional allogeneic HSCT using a conditioning regimen with high doses of systemic chemotherapy/radiation is associated with significant toxicities. In contrast, HSCT with a reduced-intensity conditioning regimen allows older patients and those who have contraindicating comorbidities to undergo HSCT [2-7].

Nevertheless, special consideration should be paid to developing reduced-intensity conditioning protocols for the unrelated-donor HSCT setting, because the incidences of both graft rejection and graft-versus-host disease (GVHD) are greater than in related-donor transplantation. In addition, the intensity of the reduced-intensity conditioning regimen influences transplantation-related toxicities and the relapse rate, and the stem cell source (ie, peripheral blood stem cells or bone marrow cells) influences engraftment [8]. Accordingly, several reduced-intensity conditioning protocols have been tested to address a variety of problems [8-17]. In this study, we investigated the feasibility of bone marrow transplantation (BMT) from a serologically HLA-matched unrelated donor with a regimen containing

Correspondence and reprint requests: Yoichi Takaue, National Cancer Center Hospital, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104-0045, Japan; 81-3-3542-2511; fax: 81-3-3542-3815 (e-mail: ytakaue@ncc.go.jp).

4 Gy of total body irradiation (TBI), fludarabine (Flu), and busulfan (BU).

2. Patients and Methods

2.1. Patients and Donors

The data for adult patients with hematologic malignancies who underwent unrelated-donor BMT through the Japan Marrow Donor Program between June 2002 and December 2003 at the National Cancer Center Hospital were analyzed retrospectively. This protocol was approved by the Ethics Committee, and written informed consent was obtained from each patient. The patients who were enrolled in this study were ineligible for conventional allogeneic HSCT because of age (older than 50 years) and/or concomitant diseases or preceding intensive therapies, such as autologous HSCT or multiple chemotherapies. Donor-recipient pairs were selected on the basis of serologic matching for HLA-A and HLA-B and molecular matching for HLA-DRB1. HLA allele typing was performed by intermediate-resolution polymerase chain reaction (PCR) analysis. The stem cell source, which was determined by the Japan Marrow Donor Program donor center, was bone marrow in all cases.

2.2. Treatment Plan and Evaluations

The conditioning regimen consisted of 30 mg/m^2 Flu intravenously daily for 6 days (day –8 to day –3), 4 mg/kg BU orally daily for 2 days (days –6 and –5, without BU dose adjustment), and 4 Gy TBI without lung shielding (day –9 or day –1, single dose or 2 divided doses). Non–T-cell–depleted bone marrow was infused on day 0. The time of neutrophil engraftment was defined as the first of 3 consecutive days with an absolute neutrophil count $\geq 0.5 \times 10^9$/L, and the time of platelet engraftment was defined as the first of 7 consecutive days with a platelet count $\geq 20 \times 10^9$/L without transfusion support. Granulocyte colony-stimulating factor (G-CSF) was administered at 300 μg/m^2 from day 6 and continued until neutrophil engraftment. The degree of donor chimerism among peripheral blood mononucleated cells was evaluated by PCR analysis of short tandem repeat polymorphisms with fluorescently labeled primers. Secondary graft failure was defined as cytopenia with an absolute neutrophil count $< 0.1 \times 10^9$/L or decreasing chimerism not associated with relapsing disease in patients who had recovered in the early posttransplantation period.

GVHD prophylaxis consisted of cyclosporin A (CsA) from day –1 (daily administration of 3 mg/kg by continuous intravenous infusion or 6 mg/kg orally in 2 divided doses) and methotrexate (10 mg/m^2 intravenously on day 1 and 7 mg/m^2 on days 3, 6, and 11). The CsA dosage was adjusted according to the patient's renal function and to maintain therapeutic levels (250-350 ng/mL) with continuous infusion or trough levels (150-250 ng/mL) with oral administration. In patients without GVHD, CsA was tapered from day 100 over a 3- to 6-month period. Standard criteria were used to grade acute and chronic GVHD [18,19]. Chronic GVHD was evaluated in patients who survived at least 100 days and was classified as limited or extensive. Patients who developed acute GVHD ≥grade II were treated with methylprednisolone at 1 to 2 mg/kg per day.

2.3. Supportive Care

Antimicrobial prophylaxis consisted of ciprofloxacin, fluconazole, acyclovir, and trimethoprim/sulfamethoxazole according to our institutional protocol. All patients were nursed in a room equipped with high-efficiency air filtration of particulates. Monitoring for cytomegalovirus (CMV) antigenemia was performed once a week after neutrophil engraftment by means of the horseradish peroxidase–C7 method. Patients positive for CMV antigenemia were started preemptively on ganciclovir therapy.

2.4. Statistical Analysis

Overall survival was calculated from the time of transplantation until death from any cause. Progression-free survival was measured from transplantation until disease progression or death from any cause. Nonrelapse death was defined as death due to any cause other than relapse. Survival curves for overall survival and progression-free survival were estimated by the Kaplan-Meier method.

3. Results

3.1. Patients

The median age of the 17 patients was 55 years (range, 27-67 years; Table 1). The diagnoses were acute myeloid leukemia (AML) (n = 7), myelodysplastic syndrome (MDS) (n = 4), chronic myelogenous leukemia (n = 1), non-Hodgkin's lymphoma (n = 4), and multiple myeloma (n = 1). Six patients were in remission at transplantation, and the remaining 11 were not in remission. Three patients with MDS or AML following MDS underwent unrelated-donor BMT as a primary treatment. Seven donor-recipient pairs were fully matched for HLA-A, HLA-B, and HLA-DRB1 at the allele level, 4 donor-recipient pairs had an allele-level mismatch at the HLA-A locus, and 5 pairs had an allele-level mismatch at the HLA-DRB1 locus. One patient was mismatched with the donor at 3 HLA alleles.

3.2. Engraftment and Chimerism

The median number of infused nucleated cells was 2.7×10^8/kg (range, $0.65\text{-}5.5 \times 10^8$/kg). All patients achieved neutrophil recovery, but 5 patients did not become independent of platelet transfusion during their follow-up period (Table 2). The median times until neutrophil and platelet recoveries were 18 days (range, 14-35 days) and 26 days (range, 15-112 days), respectively (Figure 1). Late graft failure was observed in 2 patients, one of whom had secondary graft failure due to myelosuppression caused by ganciclovir treatment for CMV colitis. In this patient, donor chimerism was not assessed after day 30 when complete donor chimerism was confirmed. In the other case, donor chimerism decreased from 89% on day 30 to 33% on day 60, despite the tapering of CsA from day 30. Chimerism was

Table 1.
Patient and Disease Characteristics*

Patient No.	Age, y/Sex	Disease	Status	Time from Dx to HSCT, mo	HLA Allelic Mismatch	GVH Vector	HVG Vector	Contraindications to Conventional HSCT	Pretransplantation Comorbidities
1	55/F	AML	CR3	117				Age	No
2	52/F	AML	Primary Ref	13	DRB1	1	1	Age + comorbidity	Pneumonia
3	57/F	AML	Rel2	28				Age	Atrial fibrillation
4	55/M	MDS	Primary Ref	3				Age	Atrial fibrillation
5	57/M	MDS	CR1	8				Age	No
6	59/M	CML	CP2	8				Age	No
7	55/M	PTCL	PR	16	DRB1	1	1	Age	Gastric ulcer
8	58/M	AML	Untreated	10	DRB1	1	1	Age	Bronchial asthma, FEV_1 75%
9	59/M	AML	Untreated	33	DRB1	1	1	Age	Bilirubin 1.5 mg/dL
10	52/M	AML	CR1	11	A	1	1	Age	FEV_1 67%
11	57/M	MDS	CR1	13				Age	Prior gastric cancer
12	61/M	AML	CR2	58	A, both DRB1	3	3	Age	No
13	67/F	FL	Primary Ref	58	A	1	1	Age + comorbidity	Dyspnea requiring oxygen
14	27/M	DLBCL	Rel3	38	A	1	0	Prior autologous HSCT	No
15	48/F	MM	Primary Ref	80				Comorbidity	Ventricular septal defect
16	52/F	MDS	Untreated	130	A	1	1	Age	No
17	49/M	FL	Rel1	28	DRB1	1	1	Prior multiple chemotherapies	No

*Dx indicates diagnosis; HSCT, hematopoietic stem cell transplantation; GVH, graft-versus-host; HVG, host-versus-graft; AML, acute myeloid leukemia; CR3, third complete remission; Ref, refractory; Rel2, second relapse; MDS, myelodysplastic syndrome; CML, chronic myelogenous leukemia; CP2, second chronic phase; PTCL, peripheral T-cell lymphoma; PR, partial remission; FEV_1, forced expiratory volume in 1 second; FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma; MM, multiple myeloma.

evaluated by analysis of short tandem repeats in 14 patients, and complete donor chimerism was confirmed in 12 of these patients. One patient who relapsed on day 32 had exhibited 54% donor chimerism on day 30. In the remaining 3 patients who relapsed after transplantation, complete donor chimerism had been achieved by day 30. In the patient who relapsed on day 78, donor chimerism decreased from 100% on day 30 to 64% on day 60. Mixed chimerism was not confirmed in the other 2 patients before disease progression or relapse. The patients without graft failure or relapse did not have mixed chimerism during their follow-up periods.

3.3. Regimen-Related Toxicities and Infections

Regimen-related toxicity was graded according to the National Cancer Institute Common Toxicity Criteria, version 2.0, and maximum toxicities are shown in Table 3. Fifteen of the 17 patients had grade III oral/pharyngeal mucositis that required morphine as an analgesic. Reversible elevation (grades III-IV) in transaminase and bilirubin levels occurred in 35% and 12% of the cases, respectively. No veno-occlusive disease was observed. Four patients developed transient grade III hyponatremia within 28 days after transplantation. Four patients developed transient pulmonary infiltration or congestive heart failure due to hypercytokinemia at engraftment, and 2 of these patients developed grade II acute GVHD after engraftment. No histologic findings of acute GVHD were seen in the other 2 patients. One patient developed reversible paroxysmal supraventricular tachycardia. One patient developed bloody diarrhea and abdominal pain even after improvement of acute GVHD of the skin, and we diagnosed intestinal thrombotic microangiopathy from the results of a gut biopsy. This patient was successfully managed by diminishing immunosuppressive treatment. Four patients who had blood cultures positive for bacterial infection (*Pseudomonas aeruginosa*, *Acinetobacter lwoffii*, *Corynebacterium* sp, and *Staphylococcus* sp) within 28 days after transplantation were successfully treated with antibiotics. Invasive aspergillosis was encountered in 2 patients (1 proven and 1 possible case). In the proven case, the patient had bronchiolitis obliterans, which was the ultimate cause of death. Of the 17 patients, CMV antigenemia was detected in 12 patients, 2 of whom had CMV colitis.

3.4. Graft-versus-Host Disease

Acute GVHD of grades II to IV was diagnosed in 8 patients (48%; 95% confidence interval [CI], 36%-59%); the GVHD was grade II in 3 patients and grade IV in 5. The median time to the onset of acute GVHD was 32 days (range, 20-81 days) after transplantation (Figure 2A). Two of 4 patients who skipped methotrexate treatment on day 11 because of severe mucositis developed grade IV acute GVHD. Two of the 5 patients with grade IV acute GVHD subsequently died. One of these patients had acute GVHD after the withdrawal of CsA treatment at the time of leukemia relapse, and the other patient had received bone