Biology of Blood and Marrow Transplantation 13:172-182 (2007)

1083-8791/07/1302-0001$32.00/0
doi:10.1016/j.bbmt.2006.09.007

Reticulocyte Maturation Parameters Are Reliable Early Predictors of Hematopoietic Engraftment after Allogeneic Stem Cell Transplantation

J. R. Molina, J. Sanchez-Garcia, A. Torres, M. A. Alvarez, J. Serrano, J. Casaño, P. Gomez, F. Martinez, A. Rodriguez, C. Martin

Hematology Department and Bone Marrow Transplantation Unit, University Hospital Reina Sofia, Cordoba, Spain

Correspondence and reprint requests: J. Sanchez-Garcia, MD, PhD, Hematology Department, University Hospital Reina Sofia, Avenida Menendez Pidal s/n, 14004 Cordoba, Spain (e-mail: joaquin.sanchez@cheerful.com).

Received July 27, 2006; accepted September 18, 2006

ABSTRACT

Early detection of donor-derived hematopoietic restoration after allogeneic stem cell transplantation (allo-SCT) is a crucial issue in the management of heavily immunocompromised patients. The aim of this prospective study was to validate our previously defined cutoff values for reticulocyte maturation parameters as early predictors of hematopoietic engraftment. Importantly, the effect of clinical variables in reticulocyte engraftment was also sought. For this purpose, we prospectively studied 136 consecutive patients undergoing allo-SCT from related (n = 89) or unrelated (n = 47) donors. High fluorescence reticulocytes (RETH), immature reticulocyte fraction (IRF), mean fluorescence index (MFI), and mean reticulocyte volume (MRV) were automatically measured in peripheral blood samples drawn on a daily basis. We previously defined reticulocyte engraftment when MFI ≥10, RETH ≥3%, IRF ≥10%, and MRV ≥110 fL. Median neutrophil engraftment was 18 days (range, 10-35 days); for reticulocyte parameters, the values were 14 days for IRF (range, 7-45 days), 14 days for MFI (range, 7-43 days), 15 days for RETH (range, 7-43 days), and 21 days for MRV (range, 9-74 days). These differences reached statistical significance for MFI and IRF when compared with standard neutrophil recovery, even when analyzing siblings or unrelated donors separately. In univariate analysis, donor-recipient ABO disparity adversely influenced erythroid engraftment ($P = .04$ for IRF, $P = .03$ for MFI), but the infusion of $>2.9 \times 10^6$/kg of $CD34^+$ cells was associated with a shorter time to reach erythroid engraftment ($P = .02$ for IRF and MFI). In Cox regression analysis, ≥100/μL neutrophils and IRF ≥10% were predictive parameters for standard neutrophil engraftment. Based on these findings, we suggest that serial measurement of IRF or MFI should be routinely used to trace hematopoietic restoration after allo-SCT because these preceded standard neutrophil recovery by a median of 4 days and are therefore very useful to make clinical decisions.

KEY WORDS

Reticulocytes • Allogeneic stem cell transplant • Engraftment

INTRODUCTION

Allogeneic stem cell transplantation (allo-SCT) has become the only curative treatment for selected patients who have mainly neoplastic hematologic disorders. The increasing use of alternative donors other than HLA-identical siblings and the use of cord blood units even in adults are associated with a delayed engraftment and increased graft failure rates up to 20% [1,2]. Prolonged neutropenic periods after allo-SCT are associated with an important risk of life-threatening infections, which are the most common cause of transplant-related mortality [3], and any sign of oncoming neutrophil recovery would be very useful in the management of these patients. Over the past few years, some biological parameters have been tested as early predictors of hematopoietic recovery [4-6], but measurement of peripheral blood reticulocytes has been the most attractive surrogate marker [7]. Hematopoietic restoration after marrow ablation by conditioning regimens is heralded by the erythroid compartment because the most differentiated erythroid cells are easily allowed to pass through the bone

marrow sinusoid cells. The introduction of automatic flow cytometric methods for measuring these erythroid cells has greatly improved the precision and accuracy of reticulocyte counting and has currently replaced manual-visual counting techniques [8-10]. Flow cytometry has also let us to obtain reticulocyte maturation parameters based on reticulocyte total RNA content and volume. The erythroid compartment cells in their maturation process lose RNA and progressively shrink in volume, and thus the youngest reticulocytes contain larger amounts of RNA and larger volumes, being the first population to appear in the bloodstream during hematopoietic recovery after allo-SCT.

We and others previously tested reticulocyte maturation parameters as the earliest, easily measurable data of marrow engraftment after autologous or allo-SCT [11-15]. In these series, highly fluorescent reticulocytes (RETH), the sum of highly and medium fluorescent reticulocytes also known as immature reticulocyte fraction (IRF), mean fluorescence index (MFI), and mean reticulocyte volume (MRV) have proved useful before neutrophil engraftment within a range of 2 of 6 days. Other calculated parameters such as time to doubling IRF [16] or the ratio of MRV to mean corpuscular volume (MCV) [13] could precede neutrophil recovery by even up 9 days before engraftment. However, the widespread, routine use of these reticulocyte maturation parameters as predictors of engraftment after allo-SCT has been limited by the fact that none of them have been validated in large prospective series. Another important drawback is the lack of well-defined stringent and uniformed engraftment criteria for each reticulocyte maturation parameter among different studies [11-16].

In a pilot series of 30 consecutive patients undergoing allo-SCT and 30 undergoing autologous SCT, we applied a statistical approach to stringently define cutoff points as markers of reticulocyte engraftment [11]. In the present study, we prospectively evaluated these pre-established cutoffs as early predictors of hematopoietic recovery but with a focus on allo-SCT procedures, which are associated with longer neutropenia periods and a higher incidence of graft failure compared with autologous transplantations. In this large consecutive series of allo-SCT, we include patients who received grafts from HLA-identical sibling donors and from unrelated and mismatched donors. We also analyzed several clinical variables that could have influenced red cell engraftment and their behavior in cases of graft failure.

METHODS

Patients

Patients (n = 136) who underwent allo-SCT at the University Hospital Reina Sofia (Cordoba, Spain) between January 2000 and May 2005 were included in this study. Clinical data are summarized in Table 1.

Table 1. *Clinical Characteristics of 136 Patients with Allogeneic SCT*

Parameters	Number	%
Number of total patients	**136**	
Age (y) at SCT, median (range)	**33 (1-64)**	
Male/female	**81/55**	**59.5/40.5**
Diagnoses		
Acute leukemia	**68**	**50**
Chronic myeloid leukemia	**30**	**22**
Multiple myeloma	**12**	**8.8**
Marrow aplasia	**11**	**8.1**
Non-Hodgkin lymphoma	**7**	**5.2**
Other	**8**	**5.9**
Donor type		
Sibling donor*	**88**	**64.7**
Unmatched donor†	**47**	**34.5**
Haploidentical related‡	**1**	**0.7**
ABO compatibility		
Compatible	**87**	**64**
Minor incompatibility	**21**	**15.4**
Major incompatibility	**28**	**20.6**
Graft source		
Bone marrow	**113**	**83**
Peripheral blood	**14**	**10.3**
Umbilical cord	**9**	**6.6**
Preparative regimen		
Myeloablative with TBI	**94**	**69.1**
Myeloablative without TBI	**23**	**17**
Nonmyeloablative	**8**	**5.9**
CY + ATG	**11**	**8**
GVHD prophylaxis		
CsA + MTX	**118**	**87**
MMF + CsA	**8**	**5.9**
CsA + PDR	**9**	**6.6**
Mean CD34 infused cells ($\times 10^6$/kg)	**2.9**	
Mean mononuclear infused cells ($\times 10^8$/kg)	**2.6**	

SCT indicates stem cell transplantation; TBI, total body irradiation (13 Gy); CY, cyclophosphamide (120 mg/kg); ATG, antithymocyte globulin; PDR, prednisone; CsA, cyclosporine (3 mg/kg); MTX, methotrexate (10 mg/m^2); GVHD, graft-versus-host disease; MMF, mycophenolate mofetil (30 mg/kg).

*Five sibling donors were mismatched by 1 or 2 antigens.

†Fourteen unrelated donors were mismatched by 1 or 2 antigens including all 9 umbilical cord units.

‡Patient grafted from haploidentical sibling donor was conditioned with TBI, fludarabine, and tiothepa and received purified peripheral blood CD34 cells without further GVHD prophylaxis.

Allo-SCTs were performed from related (n = 89) or unrelated (n = 47) donors who were serologically or allele tested for HLA-A, -B, and -C and were allele tested specifically for HLA-DRB1 and DQB1. Nineteen patients received grafts from donors with 1 or 2 minor disparities (5 from sibling donors and 14 from unrelated donors) and 1 patient from a haploidentical sibling donor. Eight patients received a nonmyeloablative conditioning regimen because of age or associated comorbidity. Stem cell source was bone marrow (n = 113), mobilized peripheral blood (n = 14), or cord blood (n = 9). Prophylaxis against graft-versus-host disease (GVHD) for patients undergoing myeloablative allo-SCT consisted mainly of a short course of methotrexate (MTX) at a dose of 10 mg/m^2

on days +1, +3, +6, and +11 plus cyclosporine (CsA) at a dose of 1.5 mg/kg intravenously or 6.25 mg/kg orally every 12 hours on days −1 to +90 that was then tapered until day +180. Prophylaxis against GVHD for patients undergoing nonmyeloablative allo-SCT consisted of mycophenolate mofetil (MMF) at a dose of 15 mg/kg orally every 12 hours from day 0 to day 40 with subsequent tapering until day 90 and CsA at a dose of 1.5 mg/kg intravenously or 6.25 mg/kg orally every 12 hours on days −1 to +90 and then tapered until day +180. All patients received granulocyte-colony stimulating factor at a dose of 5 μg/kg daily, starting 7 days after graft infusion until they achieved for 3 consecutive days a granulocyte peripheral blood count >500/μL. All patients received packed red blood cell transfusions to maintain hemoglobin concentrations >8 g/dl (≥25% hematocrit) and packed random or single-donor platelets were transfused when platelet counts decreased to $<20 \times 10^9$/L. One hundred twenty-seven patients received prophylaxis with 500 mg/m^2 acyclovir intravenously during the neutropenia period. All received antifungal and antibiotic prophylaxis (with fluconazole or voriconazole and ofloxacin, respectively) since the first day of the conditioning regimen. No patient received erythropoietin during the transplantation course. During the study period, no clinical interventions were taken based on reticulocyte maturation parameters.

Methods

Reticulocyte analysis was performed with the ABX PENTRA 120 Retic (Horiba-ABX, Montpellier, France). Briefly, reticulocyte counting requires a preliminary manual mixing 0.8 μL of whole peripheral blood with ethylenediaminetetraacetic acid with 2.5 mL of a proprietary formulation of nucleic acid of fluorochrome thiazole orange. After 25 seconds of incubation at 35°C, an aliquot of dilution was transferred to the optical bench, and cells were analyzed sequentially to determine the true MRV by aperture of impedance (resistivity) and fluoro-flow cytometry (RNA content) using a 20-mW argon ion laser light source. Using customized gating for each sample, reticulocytes are separated from mature red blood cells, white blood cells, and platelets. The results are displayed on a reticulocyte matrix with RNA content on the y axis and cell volume on the x axis. For each peripheral blood sample, the analyzer obtains reticulocyte percentage and absolute reticulocyte count. Moreover, because the amount of light absorbed by reticulocytes is proportional to the intensity of staining and RNA content, reticulocytes are subdivided into 3 populations with low, medium, and high RNA content. With this subdivision we obtained reticulocytes in 3 sections: low RNA content, medium RNA content (RETM) and high RNA content (RETH). The immature reticulocyte fraction is a parameter calculated with the sum of RETM plus RETH. The other 2 parameters, MFI and MRV, were calculated directly by the analyzer.

All values were determined from peripheral blood samples drawn the day before the conditioning regimen, the infusion day (designated day 0), and thereafter on a daily basis until neutrophil counts remained > 500/μL for 3 days. All samples were run in duplicate and results are presented as mean values. Engraftment was defined as an absolute neutrophil count (ANC) of 500/μL for 3 consecutive days and a platelet count of 20×10^9/L in peripheral blood and independent of red blood cell transfusion.

Statistical Analysis

Results are expressed as mean ± standard deviation and range for reticulocyte values and median for engraftment days. Highly and medium fluorescent reticulocytes are expressed as percentages, MFI as fluorescence units, and MRV in femtoliters. We proposed a statistical model indicating erythroid engraftment defined by each reticulocyte variable: the first post-transplantation day when MFI values reach ≥10, IRF values reach ≥10%, RETH values reach ≥3%, and MRV values reach ≥110 fL for each patient for ≥3 consecutive days [11]. These cutoffs were assigned considering the adjusted 25% quartile for each parameter on the day that myeloid engraftment occurred based on our previous data. These were compared with ANCs ≥100/μL and ≥500/μL (the first day these peripheral blood counts were achieved).

Temporal series Kaplan-Meier and log-rank tests were used to compare days of engraftment for reticulocyte parameters with days of ANC engraftment. The relation between different reticulocyte parameters and myeloid engraftment was estimated by simple linear regression and correlation analysis. The effect of clinical variables that could influence erythroid engraftment was examined by univariate and multivariate analyses using Cox regression models. $P < .05$ was accepted as statistically significant.

RESULTS

In our consecutive series of 136 patients undergoing allo-SCT, we observed 5 (3.7%) cases of primary graft failure and 5 patients died during the neutropenia period without hematopoietic recovery. The remaining 126 patients (92.6%) fulfilled engraftment criteria with donor-derived hematopoiesis as assessed by molecular chimerism analyses. Eight patients who underwent allo-SCT with nonmyeloablative conditioning regimen showed a transient mixed chimerism with 2.4% (range, 1.1%-5.7%) of recipients' marrow nucleated cells measured at day +17 (range, 14-20 days). After the conditioning regimen, absolute reticu-

Table 2. *Median (Range) Number of Days to Reach Reticulocyte Maturation Parameter Engraftment, Monocyte and Standard Neutrophil Engraftment in 126 Patients with Successful Donor-Derived Hematopoietic Restoration after Allogeneic SCT*

Patients (n)	IRF-DT*	MFI ≥10	IRF ≥10%	AMC ≥100/μL	ANC ≥100/μL	ANC ≥500/μL	ANC ≥1000/μL
All (126)	11 (4-25)	14 (7-43)	14 (7-45)	15 (7-27)	15 (9-37)	18 (10-35)	19.5 (10-35)
SD (87)	11 (4-23)	14 (7-28)	14 (7-26)	15 (9-27)	15 (9-25)	17 (10-26)	19 (10-27)
URD (39)	13 (8-25)	14 (8-43)	14 (8-45)	15 (7-27)	16 (9-37)	19 (12-48)	21 (13-35)
BM (106)	11 (4-23)	14 (7-28)	14 (7-26)	15 (9-25)	15 (9.25)	18 (12-26)	20 (13-27)
UCB (8)	15 (9-25)	22 (14-43)	22 (14-45)	16 (7-27)	22 (13-37)	26 (17-48)	27 (19-35)
MPB (12)	10 (7-13)	10 (8-29)	10 (8-29)	16 (7-13)	14 (10-21)	15.5 (10-22)	16.5 (10-26)

SCT indicates stem cell transplantation; SD, sibling donor; URD, unrelated donor; BM, bone marrow; UCB, umbilical cord blood; MPB, mobilized peripheral blood; IRF-DT, immature reticulocyte fraction doubling time; MFI, mean fluorescence index; IRF, immature reticulocyte fraction; AMC, absolute monocyte count; ANC, absolute neutrophil count.

*This parameter could be applied in only approximately 50% of cases.

locyte counts decreased progressively from 115 × 10^9/L to a nadir of 6.1 × 10^9/L on median day +9. Thereafter, increasing numbers of reticulocytes and increasing percentages of immature fractions were observed throughout the recovery period.

Kinetics of Reticulocyte Maturation Parameters and Neutrophil Recovery after Allo-SCT

After the reticulocytopenia period, there was a significant increase in peripheral blood immature reticulocytes before neutrophil recovery. Median days are listed in Table 2. Thus, using our pre-established criteria, median engraftment days were +14 (range, 7-45) for IRF, +14 (range, 7-43) for MFI, +15 (range, 7-43) for RETH, and +21 (range, 9-74) for MRV. Myeloid engraftment (ANCs ≥100/μL and ≥500/μL) occurred later than IRF and MFI (day +15 [range, 9-37] and day +18 [range, 10-35], respectively). These differences reached a statistical difference for IRF ≥10% and MFI ≥10 compared with an ANC ≥500/μL ($P < .01$ in both cases) and for MFI ≥10 compared with an ANC ≥100/μL ($P = .06$). Remarkably, only 2 patients did not achieve these cutoff values (1 eventually developed secondary graft failure), which can, therefore, be applied to 98.4% of cases. In this large series, neither RETH ≥3% nor MRV ≥110 fL reached statistical differences compared with standard neutrophil recovery. Further, 10.3% (n = 13) and 44.4% (n = 56) of patients did not reach cutoff values for RETH and MRV, respectively. Therefore, the most powerful predictors were IRF and subsequently MFI, which encompasses the fluorescence mostly provided by high and medium fluorescent reticulocytes.

When we considered patients with allo-SCT grafting from a sibling donor (n = 87), MFI reached ≥10 at day +14 (range, 7-28), whereas ANC ≥100/μL and ≥500/μL occurred significantly later ($P = .04$ and $P < .01$, respectively). Likewise, IRF ≥10% was reached at day +14 (range, 7-26), which was significantly sooner than an ANC ≥500/μL ($P < .01$). More interestingly, patients undergoing allo-SCT from unrelated donors (n = 39) also achieved MFI values ≥10 and IRF values ≥10% before standard neutrophil recovery with an ANC ≥500/μL ($P = .02$ and .05 for MFI and IRF, respectively). Kaplan-Meier curves comparing reticulocyte maturation engraftment with ANC engraftment for the global series and according to donor type are displayed in Figures 1 and 2.

We tested all parameters that could potentially predict an eventual hematopoietic restoration in all consecutive 136 patients. In Cox regression analysis, only 2 parameters were absolutely predictive of myeloid engraftment: ANC ≥100/μL and IRF ≥10%. Notwithstanding, it is important to highlight that an IRF >10% occurred 1-4 days sooner than an ANC ≥100/μL in most instances.

In an autologous transplantation setting, it has been stated that other calculated parameters, ie, the IRF doubling time (IRF-D), as the first of 2 consecutive days on which the IRF value doubled from the nadir, can precede an ANC ≥100/μL by several days before. In our series of allo-SCT, this parameter could not be stringently applied because in 59 patients (47.2%) the nadir value of IRF was virtually 0. For the remaining 67 patients, IRF-D occurred significantly sooner than an ANC ≥100/μL ($P < .01$).

An absolute monocyte count (AMC) ≥100/μL was also tested as an early predictor of eventual hematopoietic restoration. This cutoff value was reached in most patients (95.2%). Median day of an AMC ≥100/μL occurred significantly sooner than the standard ANC ≥500/μL ($P = .02$, log-rank test), but no statistical difference was found when compared with an ANC ≥100/μL ($P = .3$). Importantly, median days of MFI values ≥10 and IRF values ≥10% occurred significantly sooner than an AMC ≥100/μL ($P = .009$ and .034, log-rank test, for MFI and IRF, respectively). Median days are presented for all early predictor parameters in Table 2.

Analysis of Clinical Factors Influencing Reticulocyte Engraftment

We identified IRF and MFI as statistically significant early predictors of hematopoietic recovery when

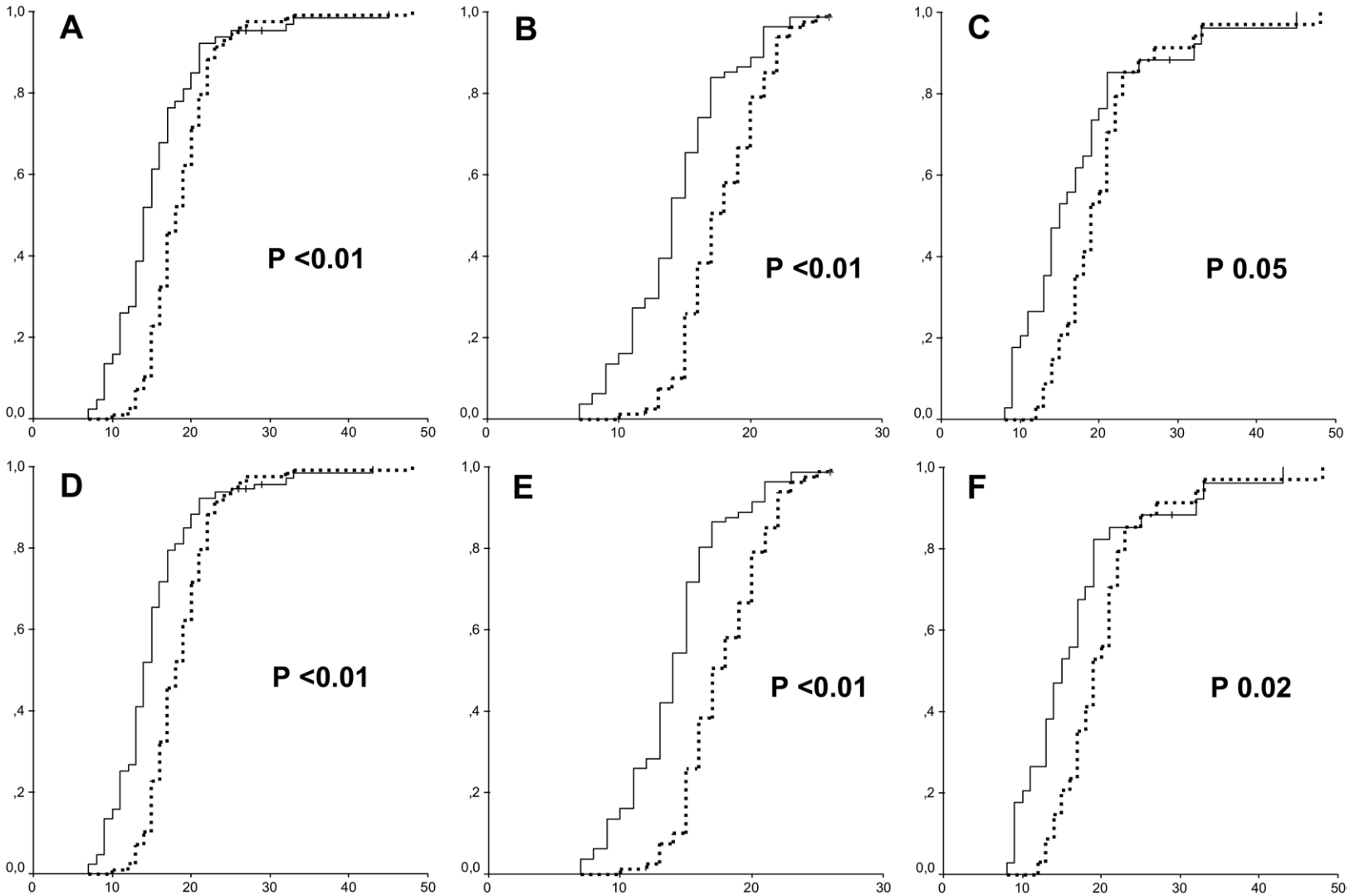

Figure 1. Kaplan-Meier curves comparing reticulocyte engraftment days and standard absolute neutrophil count (ANC) engraftment (≥500/μL) after allogeneic transplantation. Days after transplantation are presented on the x axis and cumulative frequency of achieving engraftment is presented on the y axis; solid lines represent reticulocyte maturation parameters for engraftment and dashed lines represent standard ANC engraftment. Immature reticulocyte engraftment (IRF; ≥10%) is compared with ANC in (A) all patients with successful engraftment, (B) patients who received grafts from a sibling donor, and (C) patients who received grafts from an unrelated donor. Mean fluorescence index (MFI; ≥10) is compared with ANC in (D) all patients with successful engraftment, (E) patients who received grafts from a sibling donor, and (F) patients who received grafts from an unrelated donor.

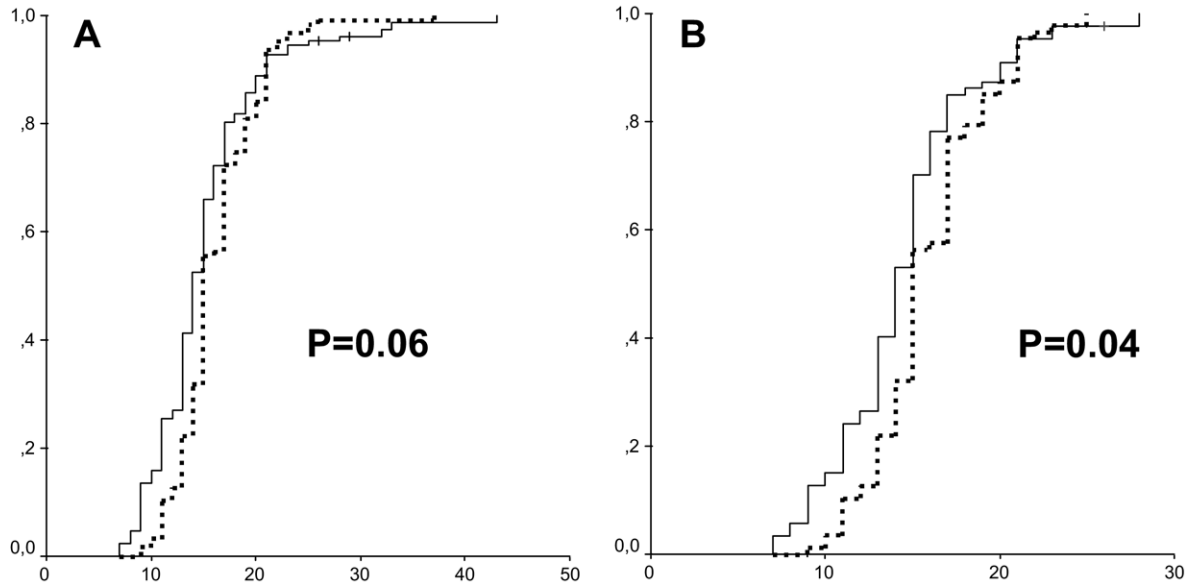

Figure 2. Kaplan-Meier curves comparing mean fluorescence index (MFI) engraftment (≥10) days and more stringent absolute neutrophil count (ANC) engraftment day (≥100/μL) after allogeneic transplantation. Days after transplantation are presented on the x axis and cumulative frequency of achieving engraftment is presented on the y axis; solid lines represent MFI engraftment and dashed lines represent day of achieving an ANC ≥100/μL. (A) All patients with successful engraftment and (B) patients who received grafts from a sibling donor.

compared with an ANC ≥500/μL in patients who received grafts from a sibling donor or those who received grafts from an unrelated donor. We also sought other clinical factors that could influence reticulocyte engraftment.

As expected, reticulocyte engraftment and neutrophil recovery were heavily influenced by stem cell source ($P < .01$, Kruskal-Wallis test for MFI, IRF, and ANC). Median engraftment days are listed in Table 2, and the earliest are recorded for the mobilized peripheral blood group and the latest for the UCB group. In either case, IRF and MFI precede an ANC ≥500/μL by a minimum of 4 days, differences that retained statistical significance for the bone marrow grafted group (Figures 3 and 4). Therefore, IRF and MFI can be used to predict oncoming hematopoietic recovery after allo-SCT regardless of stem cell source.

The presence of minor ABO disparity had no effect on reticulocyte engraftment, but the presence of major ABO disparity was an adverse factor that significantly delayed red cell engraftment as measured by reticulocyte maturation parameters. Thus, the median day to reach an IRF ≥10% and an MFI ≥10 for patients with major ABO disparity was +15 (range, 7-45) and that for ABO-compatible patients was +14 (range, 7-26; $P = .049$ and .032 for IRF and MFI, respectively). The group of major ABO-compatible patients with successful engraftment (n = 24) included 5 patients grafted with umbilical cord blood (UCB) and 11 patients from unrelated donors. Even in this subset of patients, median engraftment days for MFI and IRF preceded by 2.5 days the median engraftment day for an ANC ≥500/μL (+17.5; range, 7-48), although without reaching statistical significance.

The number of infused $CD34^+$ cells had statistical significance in engraftment, which occurred sooner if infusion of $CD34^+$ cells was $>2.9 \times 10^6$/kg ($P = .02$ for IRF and MFI). This parameter also influenced standard neutrophil engraftment and thus an ANC ≥500/μL was reached significantly sooner in patients infused with $>2.9 \times 10^6$/kg.

Other clinical variables such as conditioning regimen, status of disease at transplantation, number of red cell transfusions, acute GVHD of any grade, herpes infectious disease, and days of fever after allo-SCT had no statistical influence on reticulocyte engraftment. The use of a short course of methotrexate as part of acute GVHD prophylaxis did not result in a delayed reticulocyte engraftment as measured by MFI and IRF values ≥10 ($P = .33$ and .44).

Table 3 presents results of univariate analysis. In Cox regression multivariate analysis, none of the clinical variables studied remained statistically significant for reticulocyte engraftment as measured by MFI or IRF.

Reticulocyte Maturation Parameters in Graft Failure Cases

When we studied 126 patients who had achieved a successful donor-derived hematopoietic restoration, an ANC ≥500/μL was reached at day +26 in 96% of cases. Remarkably, an MFI ≥10 was reached at day +23 in 96.5% of cases. Therefore, patients who did not achieve an MFI value ≥10 at day +23 were very likely to develop engraftment failure. When consider-

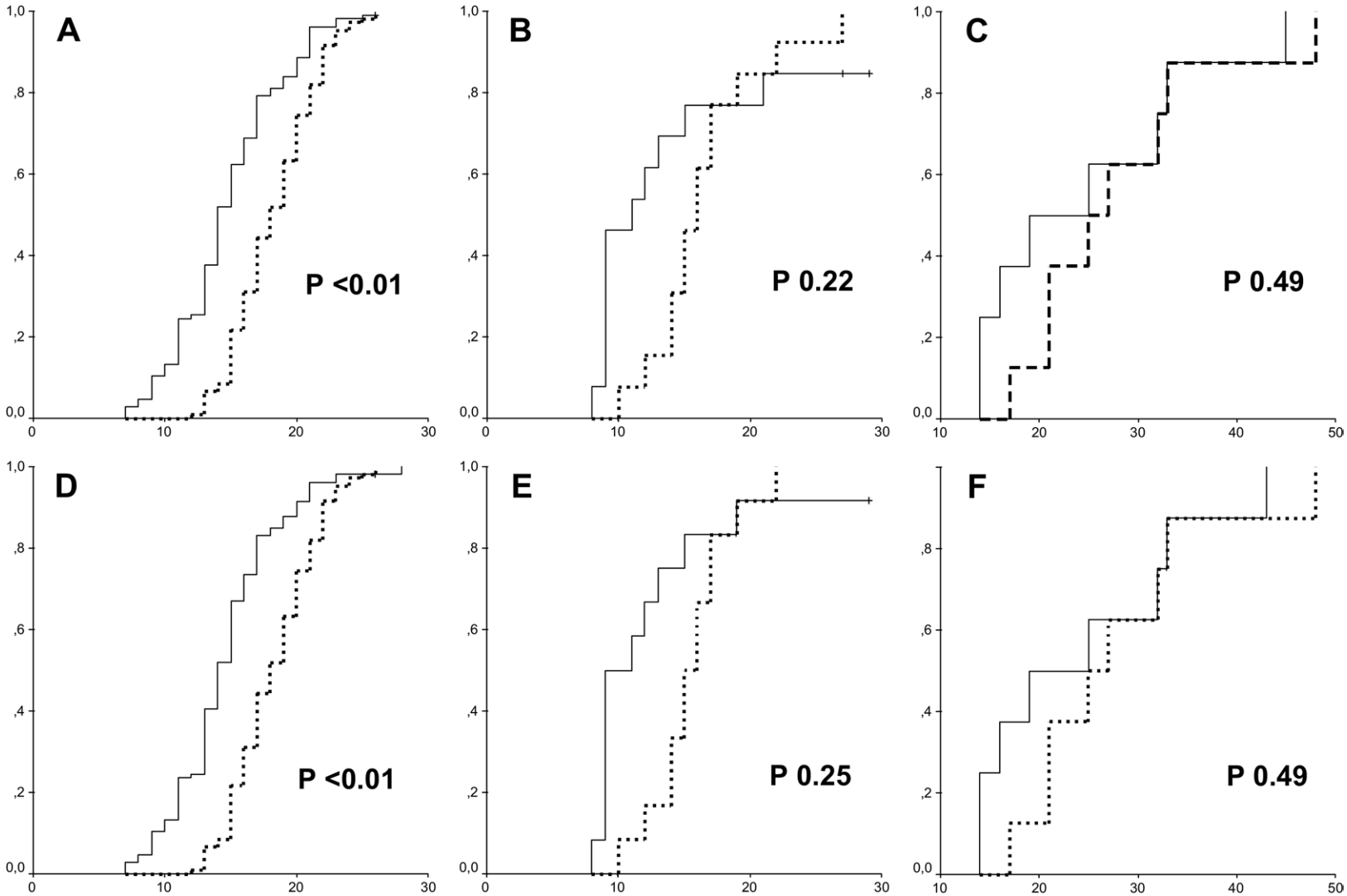

Figure 3. Kaplan-Meier curves comparing reticulocyte engraftment days and standard absolute neutrophil count (ANC) engraftment (≥500/μL) after allogeneic transplantation according to stem cell source. Days after transplantation are presented on the x axis and cumulative frequency of achieving engraftment is presented on the y axis; solid lines represent reticulocyte maturation parameter engraftment and dashed lines represent standard ANC engraftment. (A-C) Immature reticulocyte engraftment (≥10%) and (D-F) mean fluorescence index (≥10) values are presented for (A, D) bone marrow, (B, E) mobilized peripheral blood, and (C, F) umbilical cord blood.

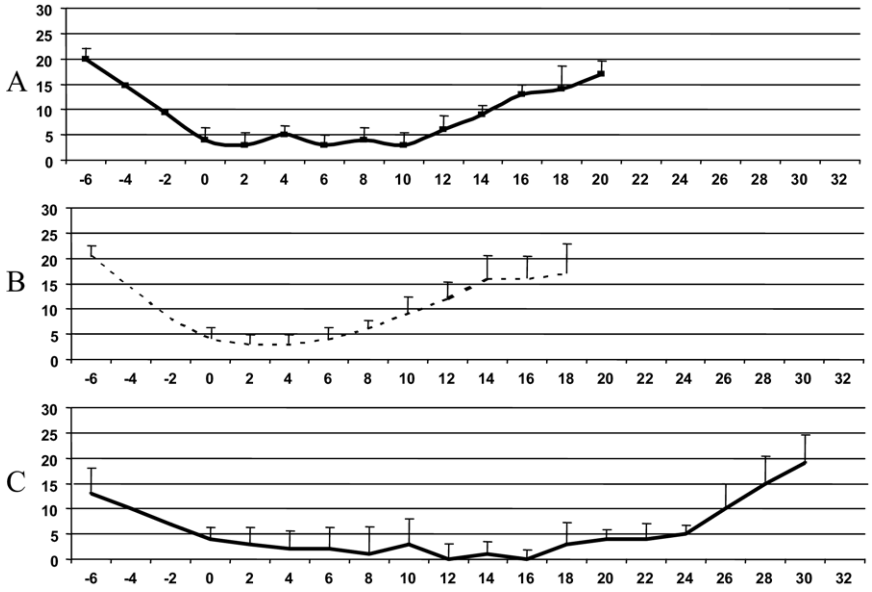

Figure 4. Kinetic immature reticulocyte engraftment measurement after allogeneic stem cell transplantation for patients grafted with (A) bone marrow, (B) mobilized peripheral blood, or (C) umbilical cord blood. Days before and after stem cell infusion are presented on the x axis, and mean ± SE immature reticulocyte engraftment percentages is presented on the y axis.

ing bone marrow as the stem cell source, most patients (96.2%) reached an MFI value ≥10 at day +21, but this value was reached sooner (day +19) for mobilized peripheral blood and later for UCB (day +33).

In this series, we observed 8 cases of engraftment failure (5.8%). Five patients developed primary graft failure and 3 patients did so after transient successful neutrophil engraftment. Clinical data of these patients are presented in Table 4. All cases of primary graft failure showed no data of reticulocyte engraftment. In 1 of 3 patients with secondary graft failure, neither MFI nor IRF values reached ≥10 after allo-SCT. Six patients received rescue stem cell infusion (5 from allogeneic donors), with complete hematopoietic restoration in 2 cases.

The first such case concerned a 42-year-old man who underwent allogeneic marrow transplantation from an HLA-matched unrelated donor for chronic myelogenous leukemia. He was infused with 2.5×10^6 $CD34^+$ cells/kg and presented a modest increase in reticulocyte parameters on day +14 without reaching cutoff values, and neutrophil counts reached up to 0.23×10^9/L. Subsequently, there were progressive decreases in reticulocyte parameters and neutrophil counts. An autologous peripheral stem cell backup graft, containing 1.37×10^7 mononuclear cells/kg, was infused on day +36 with hematopoietic recovery and complete resolution of the febrile episode.

The second case concerned a 16-month-old infant diagnosed with acute lymphoblastic leukemia. She underwent allogeneic bone marrow transplantation from a HLA-identical unrelated donor and was infused with 3.4×10^6 $CD34^+$ cells/kg. On day +18 MFI and IRF reached their cutoff values, whereas neutrophils increased to 0.5×10^9/L on day +23. Two days later (+25) reticulocyte maturation parameters and neutrophil peripheral blood counts progressively decreased. On day +49, she received a second stem cell infusion (3.15×10^6 $CD34^+$ cells/kg) from the same donor after administration of antithymocyte globulin, with sustained erythroid and myeloid recoveries on day +21 after the second infusion.

Table 3. *Results of Univariate Analysis to Study the Effect of Clinical Variables in Reticulocyte and Neutrophil Engraftment*

	P		
Clinical Variable	**MFI**	**IRF**	**ANC**
Donor type	**0.06**	**0.05**	**0.02**
Stem cell source*	**0.002**	**0.001**	**0.000**
ABO compatibility†	**0.032**	**0.049**	**0.152**
$CD34^+$ cells infused‡	**0.026**	**0.022**	**0.008**
Acute GVHD	**0.111**	**0.113**	**0.342**
RT in conditioning regimen	**0.621**	**0.587**	**0.352**

MFI indicates mean fluorescence index; IRF, immature reticulocyte fraction; ANC, absolute neutrophil count; RT, radiotherapy; GVHD, graft-versus-host disease.

*Kruskall-Wallis test.

†Patients with major ABO incompatibility versus those with ABO compatibility.

‡Versus $\geq 2.9 \times 10^6$/kg.

DISCUSSION

In this study, with a large series of consecutive patients, we have validated our previously defined cutoff values of ≥10 for MFI and ≥10% for IRF as unequivocal early predictors of eventual donor-de-

Table 4. *Clinical Data of 8 Patients Who Developed Graft Failure after Allogeneic SCT*

Age	Diagnose	Donor Type	Stem Cell Source	CD34 × 10^6/kg Infused	Graft Failure	Stem Cell Rescue	Day of Infusion	Outcome
42	CML	UR	BM	2.5	Primary	Auto	+36	Alive
45	CML	Sibling	BM	1.63	Primary	Allo	+44	Death
34	ALL	UR	BM	2.20	Primary	Allo	+40	Death
12	Fanconi	UR	MPB	1.85	Primary	NA	NA	Death
21	ALL	UR mismatch	UCB	0.10	Primary	NA	NA	Death
33	CML	UR	BM	1.58	Secondary	Allo	+60	Death
1	ALL	UR	BM	3.4	Secondary	Allo	+49	Alive
29	ALL	UR	MPB	4.66	Secondary	Allo	+42	Death

SCT indicates stem cell transplantation; CML, chronic myeloid leukemia; ALL, acute lymphoblastic leukemia; UR, unrelated donor; BM, bone marrow; MPB, mobilized peripheral blood; UCB, umbilical cord blood; NA, not applicable; Auto, autologous; Allo, allogeneic.

rived hematopoietic restoration after allo-SCT. Thus, we can ensure in the vast majority of patients that the ANC will reach 500/μL within a median of 4 days in advance when using our predefined cutoff values for MFI and IRF. More importantly, this finding can be applied regardless of donor type and stem cell source. In addition, we have also demonstrated that MFI and IRF engraftment occurred significantly sooner than an ANC ≥100/μL in patients who received grafts from a sibling donor.

One major obstacle to the widespread use of reticulocyte maturation parameters in the stem cell transplantation setting has been the lack of uniform criteria to define reticulocyte engraftment. Absolute value of RETH applied in previous studies [14,17] has been progressively replaced with fixed values of IRF and MFI or calculated by increasing doubling from nadir values [16-20]. In our pilot series, we used stringent statistical criteria to establish cutoff values for MRV, RETH, MFI, and IRF in a series of 30 allogeneic patients and 30 autologous patients [11]. However, in the present series of allo-SCT, RETH and MRV lacked statistical significance and nearly 40%-50% of patients did not reach the pre-established criteria (RETH ≥5% and MRV ≥110 fL). This finding reinforces the idea that global measurement of fluorescence as MFI or combined measurement of RETH and RETM (IRF) results in a better and more reliable quantification of immature reticulocytes. As consequence of results observed in this study, we suggest that an IRF value ≥10% could be the standard criterion of reticulocyte engraftment, because in the multivariate analysis this was the best predictor of eventual myeloid engraftment. In addition, this fixed IRF value of ≥10% could apply to all patients including autologous and allogeneic transplant recipients, can be widely and homogeneously applied to design prospective trials, and is measurable in a reproducible fashion in a large number of automated counters. Currently, available automated methods to measure reticulocytes are based on fluorescence, light scattering, or absorbance. It has been demonstrated that fluorescence-based methods, regardless of the type of nucleic acid used (thiazole orange, auramine O, CD4K530), have a very good linear correlation [21,22]. In addition, the application of doubling nadir values, although it is somehow arbitrary in patients with IRF values of 0 during the pancytopenic period (nearly 50% of cases in our experience), might be a good practice because successive increases from 0 or the nadir could point out a prompt reticulocyte recovery.

As expected, several factors influenced reticulocyte engraftment and standard neutrophil engraftment. In this sense, use of alternative donors, UCB as a stem cell source, and low doses of CD34 cells are associated with a significant delay in myeloid and erythroid engraftments. Nevertheless, we have demonstrated that in these instances an IRF ≥10% and an MFI ≥10 precede neutrophil engraftment. Our major concern was the effect of ABO disparity that could selectively impair reticulocyte engraftment and limit clinical use. Minor ABO disparity did not result in a significant delay when compared with ABO-compatible allo-SCT procedures but, as expected, major ABO disparity resulted in a significant delayed reticulocyte engraftment. However, IRF and MFI still precede neutrophil recovery, although by only 2.5 days instead of 4 days observed in the entire series.

Measurement of IRF and MFI or IRF-D as harbingers of oncoming neutrophil recovery is likely to add useful information on patient management mainly in 2 aspects: management of febrile neutropenia and primary graft failure. Current guidelines for the use of antimicrobial agents in neutropenic patients have advocated identifying low-risk patients who can be conservatively managed or high-risk patients who must undergo intensive diagnostic and therapeutic measures [23]. Expected resolution of neutropenia in <10 days and early evidence of bone marrow recovery are 2 pivotal factors for the low-risk assignment and both can only be accurately assessed with daily IRF determinations. Neutropenic patients with fever lasting >5 days after allo-SCT with IRF values <10% and lack of IRF-D are likely to still have prolonged neutropenia

and therefore are at a high risk of life-threatening invasive fungal infections. Thus, if clinically indicated, they might be considered candidates to receive additional treatment options such as granulocyte transfusions or to be enrolled in clinical trials of novel antifungal agents with activity against filamentous fungi [24,25]. In addition, in agreement with Grazziutti et al [16], we suggest that IRF measurement could homogeneously identify high-risk patients enrolled in clinical trials with antimicrobial drugs for febrile neutropenia, thus avoiding the confounding factor of the beneficial effect of resolving neutropenia.

Conversely, serial measurement of IRF after allo-SCT might provide very useful information when primary graft failure is suspected. The usefulness in secondary graft failure could not be assessed in this study, because reticulocyte maturation parameters were prospectively collected until standard neutrophil recovery. Primary graft failure after allo-SCT is associated with considerable morbidity and mortality related to infections and hemorrhagic complications. In most instances, an autologous stem cell backup is not available and the greatest chance of trilineage recovery with the lowest risk of GVHD seems to be accomplished with a boost of donor $CD34^+$ selected peripheral blood cells [26]. It is not clear how long to wait before rearranging a second donation, which can take an additional 1-4 weeks. Thus, as a consequence of the results of our study, we already know that >95% of patients who receive grafts from an HLA-matched donor or using bone marrow as the stem cell source should achieve an IRF ≥10% and an MFI ≥10 at day +21. Patients who received grafts from a mismatched donor, excluding those cases using UCB, should also achieve those values at day +25. Therefore, with the knowledge that <5% of patients will eventually demonstrate engraftment, we suggest early planning of stem cell rescue at those deadlines (autologous or allogeneic, according to availability) to prevent a fatal outcome. Prospective studies are needed to assess the contribution of the IRF measurement to the clinical management of patients after allo-SCT.

ACKNOWLEDGMENTS

This study was supported in part by the Asociacion Medicina e Investigacion and by a donation from the Instituto Roche Para las soluciones integrales en Salud foundation.

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