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Assessment of hematologic progenitor engraftment by complete reticulocyte maturation parameters after autologous and allogeneic hematopoietic stem cell transplantation

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Background and Objectives. Hematopoietic restoration after marrow ablation is initiated by the erythroid compartment. However, the absolute microscope counts or corrected percentage of reticulocytes have proven to be poor markers of hematopoietic engraftment. Some reports have highlighted the usefulness of automatic flow cytometry methods to determine highly fluorescent reticulocytes, or mean fluorescence index. In this series of 60 hematopoietic stem cell transplants, we sought the normal kinetics throughout the post-transplant period of the following reticulocyte maturing parameters: highly fluorescent reticulocytes (RETH), immature reticulocyte fraction (IRF), mean fluorescence index (MFI) and also mean reticulocyte volume (MRV).

Design and Methods. Sixty consecutive patients undergoing allogeneic bone marrow (30 cases) and autologous mobilized stem cell transplantation (30 cases) were studied. Parameters of reticulocyte maturation were measured every other day from the beginning of the conditioning regimen until myeloid engraftment.

Results. Nadir values for the analyzed reticulocyte parameters were found between days +4 and +7 and thereafter, increases in these reticulocyte parameters appeared earlier than the rise in neutrophils. We considered erythroid engraftment to have occurred on the day when RETH reached 3%, IRF 10%, MFI 10 and MRV 110 fL. These cut-offs were assigned considering the 25% quartile for each parameter on the day that the myeloid engraftment occurred. The median engraftment days for RETH were +9 and +16, for IRF +9 and +13, for MFI +9 and +13 and for MRV +11 and +13 in autologous and allogeneic procedures, respectively. When compared to standard neutrophil engraftment, IRF and MFI engraftment occurred significantly earlier in all patients. Remarkably, we found a statistical correlation between the day a reticulocyte parameter reached its cut-off and the subsequent day of absolute neutrophil count (ANC) recovery for MFI after allogeneic transplants and for MRV after autologous procedures ($p < 0.001$ and $p = 0.02$, respectively). Of all the clinical parameters tested, only the number of infused CD34 cells showed a statistical influence on erythroid engraftment in autologous transplant.

Interpretation and Conclusions. Early reticulocytes appear sooner than neutrophils after both autologous and allogeneic transplants, and any determined reticulocyte parameter can reliably measure this fraction. Nevertheless, our results show that MRV and MFI cut-offs are useful for determining subsequent myeloid engraftment. These findings could be relevant to decision-making in those patients with primary graft failure heralded by an absence of increasing values of MFI and MRV, indicating very low production of reticulocytes from the graft, who could, therefore, benefit from earlier rescue therapy.

Key words: reticulocyte, engraftment, marrow transplantation

Allogeneic and autologous stem cell transplantations have become the treatment of choice for patients with various neoplastic and non-neoplastic disorders.[1] *Ex vivo* manipulation of stem cells is increasingly used in an attempt to diminish transplant-related complications, to make allogeneic procedures across MHC barriers possible[2] and in autologous procedures to avoid the reinfusion of tumor cells.[3] However, these graft manipulations may be accompanied by an increased rate of graft failure and therefore, any early sign of hematologic engraftment could be of great help in making important clinical decisions when standard neutrophil engraftment seems to be delayed. In this sense, it has been proven that hematopoietic restoration after myeloablative regimens is initiated by the erythroid compartment[4] because under physiologic conditions the more differentiated erythroid cells are allowed to cross through the sinusoid cells by a process of cytodiabasis. Absolute counts of erythroid cells using an optical microscope or corrected percentages of reticulocytes have been proven to be poorly accurate, but some reports have highlighted the use of automatic flow cytometry methods[5,6] which yield more precise counts and also parameters of maturation related to the total RNA content and volume. The youngest reticulocytes have the highest RNA content and largest size, and as maturation proceeds, both RNA content and volume decrease progressively. The percentage of highly fluorescent reticulocytes (RETH)[7-9] and the sum of high-

ly and medium fluorescent reticulocytes (immature reticulocyte fraction, IRF) seem to have some clinical significance. Instead of percentages, the mean fluorescence index (MFI)[10] is another precise parameter which quantifies global fluorescence intensity of the immature reticulocyte population. Despite these exciting results in small series[11,12] which are based on an unique maturation parameter, several issues remain largely to be determined, e.g. which parameter is the most reliable, what are their statistical power in predicting myeloid engraftment, the influence of clinical factors and the criteria to define erythroid engraftment, which vary widely from one series to another. Thus, different cut-off values have been applied: absolute reticulocyte counts of 15-20$\times10^9$/L[4,7,10] or, using RETH, an absolute count of 0.5$\times10^9$/L[4,8] or 2-5% of total reticulocytes.[7,9] The clinical usefulness of this exciting approach has also been hampered by the variability between different automated systems.[13]

Using the ABX PENTRA 120 Retic Blood automated reticulocyte analyzer, which provides accurate quantification and separation into maturation fractions,[13] as well as mean fluorescence index and mean reticulocyte volume, we carefully traced erythroid regeneration through different reticulocyte parameters in 30 allogeneic and 30 autologous transplant recipients, in order to evaluate reticulocyte maturation kinetics and compare them with standard neutrophil counts as a potentially better tool for predicting hematologic engraftment. We also analyzed all clinical factors influencing red cell engraftment and the clinical applicability once normal values in patients who successfully engraft have been firmly established.

Table 1. Characteristics of patients receiving an autologous peripheral stem cell transplant (PSCT).

Category	*N*
N° of patients	30
Median age (range), years	36 (10-57)
Sex: male/female	13 / 17
Diagnosis:	
Acute myeloid leukemia	4
Chronic myeloid leukemia	1
Multiple myeloma	2
Lymphoma	11
Solid tumors	12
Status at PSCT:	
1st complete remission/chronic phase	14
2nd complete remission	4
Partial remission	4
Solid tumor stage II-III	8
Median time diagnosis-PSCT (range), months	9.5 (6-209)
Median MNC $\times10^8$/kg (range)	3.41(1.1-9.9)
Median CD34 cells $\times10^6$/kg (range)	3.89 (1.5-11.2)
Conditioning regimen:	
BU + CY	5
TBY + CY	2
BEAM	11
CY + Thiotepa + Carboplatinum	7
Others	5

Design and Methods

Patients

From December 1998 to January 2000, 60 consecutive patients underwent autologous peripheral blood stem cell transplantation (n. 30) and allogeneic bone marrow transplantation (n.30) from either related or unrelated donors. Their main characteristics are summarized in Tables 1 and 2, respectively.

All patients except those who received an allogeneic transplant from a haploidentical donor were given granulocyte-colony stimulating factor (G-CSF) at a dose of 5 µg/kg daily starting 7 days after the graft infusion. Patients received packed red blood cell transfusions to maintain hemoglobin concentrations above 9 g/dL and platelets were transfused when the platelet count fell below 20$\times10^9$/L.

Methods

Reticulocyte analysis was performed with the ABX PENTRA 120 Retic (ABX, Montpellier, France). On whole blood this gives a measurement of the proportional reticulocyte count and the absolute reticulocyte count. It also provides the percentages of maturation fractions according to three classes: low RNA content (RETL), medium RNA content (RETM) and high RNA content (RETH). The immature reticulocyte fraction (IRF) is a calculated parameter which comprises the RETH+RETM percentage. The ABX PENTRA also quantifies global reticulocyte RNA content by a mean fluorescence index (MFI). Briefly, 0.8 µL of whole blood with EDTA is taken and mixed with 2.5 mL of a proprietary formulation of the nucleic acid fluorochrome thiazole orange and incubated at 35°C for 25 seconds. An aliquot of the dilution is transferred to the optical bench, and cells are analyzed sequentially to determine the true mean reticulocyte volume (MRV) by aperture of impedance (resistivity) and fluoro-flow cytometry (RNA content) using a 20 mW argon ion laser light source. Using a 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.

All values were determined at the beginning of the conditioning regimen, on infusion day (designated as day 0) and thereafter every other day until neutrophil engraftment. All samples were run in triplicate and results are given as mean values. Engraftment was defined as an absolute neutrophil count (ANC) of 0.5$\times10^9$/L, and a platelet count of of 20$\times10^9$/L.

Statistical analysis

Results are expressed as mean±standard deviation (SD) and range for reticulocyte values and medians for

Table 2. Characteristics of patients receiving an allogeneic bone marrow transplantation.

Category	*N*
N° of patients	30
Median age (range), years	27 (3-51)
Sex: male/female	20/10
Diagnosis:	
Acute lymphoblastic leukemia	8
Acute myeloid leukemia	8
Chronic myeloid leukemia	8
Multiple myeloma	1
Non-Hodgkin's lymphoma	2
Marrow aplasia	3
Status at BMT:	
1st complete remission/chronic phase	19
2nd complete remission	4
Partial remission/accelerated Phase	7
Median time Diagnosis-BMT (range), months	8 (1-192)
Median MNC $\times 10^8$/kg (range)	2.04 (1-4)
Median CD34 cells $\times 10^6$/kg (range)	3.7 (0.49-22)
Conditioning regimen:	
BU + CY	4
TBY + CY	16
Others	10
Donor type:	
HLA-identical sibling	24
HLA-identical non-related	3
Haploidentical sibling	3
ABO Compat/minor incompatibility	21/9
GVHD prophylaxis:	
CyA + MTX	24
CyA + MTX + T-cell depletion	2
Others	4

engraftment days. Highly and medium fluorescent reticulocytes are expressed as percentages, MFI as fluorescence units and MRV in fL. We propose a statistical model, indicating erythroid engraftment defined by each reticulocyte variable: the first post-transplantation day when the MFI and IRF values reach 10 and 10%, respectively, RETH was higher than 3% and the MRV was higher than 110 fL for each individual patient, for at least three consecutive days. These cut-offs were assigned considering the adjusted 25% quartile for each parameter on the day that myeloid engraftment occurred.

Temporal series Kaplan-Meier and log rank tests were used to compare days of engraftment for reticulocyte parameters between autologous and allogeneic procedures and reticulocyte parameters with ANC engraftment. The relationship between the different reticulocyte parameters and myeloid engraftment was estimated by simple linear regression and correlation analysis. The effects of variables that could influence reticulocyte engraftment were examined by multivariate analysis using Cox regression models.

Results

Kinetics of reticulocyte maturation during follow-up of autologous and allogeneic transplants

All recipients of both autologous and allogeneic transplants including those with an HLA-identical unrelated donor (n=3) and an HLA-haploidentical sibling donor (n=3) had sustained engraftment, as defined by conventional neutrophil counts. The absolute reticulocyte counts prior to the conditioning regimen were 111×10^9/L and 122×10^9/L for autologous and allogeneic transplant recipients, respectively. Absolute reticulocyte count fell progressively after the conditioning regimen to a nadir value of 7×10^9/L on day +4 for autologous procedures and on day +9 in allogeneic transplants. Thereafter, increasing numbers of reticulocytes and increasing percentages of immature fractions were observed throughout the recovery period. Figure 1 schematically represents the behavior of each maturation parameter compared to ANC and absolute reticulocyte number in autologous and allogeneic cases.

The kinetics of reticulocyte engraftment differed between recipients of allogeneic and autologous grafts. As expected, all reticulocyte parameters showed flatter increases after an allogeneic transplant, as displayed in Figure 1. Median engraftment days for RETH were +9 and +16, for IRF +9 and +13, for MFI +9 and +13 and for MRV +11 and +13 in autologous and allogeneic procedures, respectively, showing statistical significant differences ($p < 0.01$) by the log rank test in all reticulocyte parameters.

Analysis of clinical factors influencing reticulocyte engraftment

Multivariate analysis failed to find any statistically significant effect of the following clinical parameters on reticulocyte value variations in the *autograft* setting: diagnosis (solid tumors or hematologic malignancies), status at transplant (first complete remission or others), conditioning regimen and number of granulocyte-macrophage colony-forming units. However, the number of infused CD34 cells (more than 3×10^6/kg) positively influenced the day of erythroid engraftment ($p = 0.013$). In the context of *allogeneic* transplants, multivariate analysis did not reveal any statistically significant correlation between rises in reticulocyte indices and the following clinical variables: conditioning regimen, donor type, total infused mononuclear cells, acute graft-versus-host disease (GVHD) prophylaxis and ABO disparity.

Statistical correlation between reticulocyte production and myeloid engraftment

The median engraftment days for the whole series were +11 for IRF (range: 7-23), +11 for MFI (range: 4-30), +11 for MRV (range: 7-33) and +13 for RETH (range: 4-31). By contrast, conventional myeloid engraftment (ANC of 0.5×10^9/L) occurred significantly later ($p <0.001$) on day +14 (range: 9-35) as did platelet engraftment (20×10^9/L)

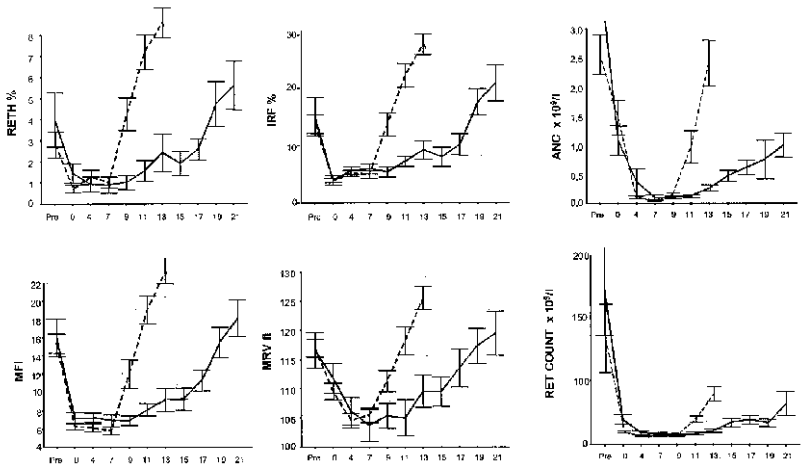

Figure 1. Mean values (± SD) of reticulocyte parameters, ANC and absolute reticulocyte counts during the post-transplant period. The x-axis represents days after the transplant. The y-axis: the bold solid line represents allogeneic procedures and the scattered line autologous procedures. RETH: highly fluorescent reticulocytes, IRF: immature reticulocyte fraction, MFI: mean fluorescence index, MRV: mean reticulocyte volume, ANC: absolute neutrophil count, Ret count: absolute reticulocyte count.

on day +15 (range: 8-53) ($p < 0.001$). When considering autologous and allogeneic procedures separately, conventional myeloid engraftment occurred on day +12 and day +16, respectively. Kaplan-Meier plots and log rank test values comparing the days of neutrophil and reticulocyte engraftment, according to each parameter, are shown in Figure 2. We tested all reticulocyte parameters to seek a possible correlation with subsequent myeloid engraftment using a multiparametric linear regression analysis, with ANC as the dependent value, using a stepwise method. MRV maintained a significance level ($p < 0.01$) in autologous transplants and MFI in the allogeneic setting ($p = 0.02$).

We also compared the day the ANC reached 0.1×10^9/L, used as an earlier indicator of myeloid engraftment, and compared this with the days of reticulocyte engraftment. Using the log rank test, only RETH engraftment occurred statistically significantly earlier than an ANC of 0.1×10^9/L in allogeneic transplant recipients ($p = 0.01$). Using multiparametric linear regression analysis, MRV and an ANC of 100×10^9/L were the predictors of subsequent standard myeloid engraftment after autologous procedures whereas only an ANC of 0.1×10^9/L remained statistically significant after allogeneic transplants.

Usefulness of reticulocyte indices in cases of graft failure

In our series of autologous transplant recipients, engraftment, as defined by MRV values of >110 fL, occurred at day +15 in 96.6% and at day +35 in 100% of studied cases. In allogeneic transplants recipients, reticulocyte engraftment, as measured by MFI >10, occurred at day +23 in 96.6% of patients and at day +32 in 100%. On the basis of this information, clinical decisions could be taken in patients with delayed neutrophil recovery, with no evidence of erythroid engraftment as shown by scoring all maturation parameters. The close monitoring of reticulocyte parameters in patient UPN 380 (not included in the series) who underwent an allogeneic marrow transplantation from an HLA matched unrelated donor for chronic myelogenous leukemia is detailed in Figure 3. This patient was infused with 2.5×10^6/kg CD34 selected positive cells. Despite a modest increase in reticulocyte parameters being observed on day +14 and neutrophils having risen to 0.23×10^9/L on day +17, there was no further significant increase above the predefined reticulocytes indices, and the neutrophil count fell again to under 0.05×10^9/L. In this situation with a prolonged febrile episode without bacterial documentation, an autologous peripheral blood stem cell back-up graft containing 1.37×10^7 cells/kg was infused on day +33, producing prompt hematologic recovery and complete resolution of the infectious episode.

Discussion

Several methods have been applied to tracing hematopoietic reconstitution after marrow ablative regimens and autologous or allogeneic rescue. These include neutrophil or platelet counts, and in the allogeneic settings, molecular techniques to detect donor hematopoiesis. Traditionally, an increasing ANC which reaches 0.5×10^9/L is referred to as proof of marrow engraftment. Notwithstanding, it has been proven that red cell engraftment occurs earlier than myeloid reconstitution, and several reports have pointed to immature reticulocytes as being the most suitable marker of this erythroid regeneration,[8,9,14-17] mainly because the standard reticulocyte percentage and absolute count show fluctuations attributable to red cell transfusions. However, if maturity indices are taken into account, the presence of immature reticulocytes indicates resumption of erythropoiesis after marrow ablation. This fraction is reliably measured by either the percentage of highly fluorescent reticulocytes, which has been previously reported to be a marker of hematopoietic engraftment,[7-9,12] or by the use of the reticulocyte maturity index.[10] Neither of these two parameters has been standardized as truly predictive of hematopoietic engraftment

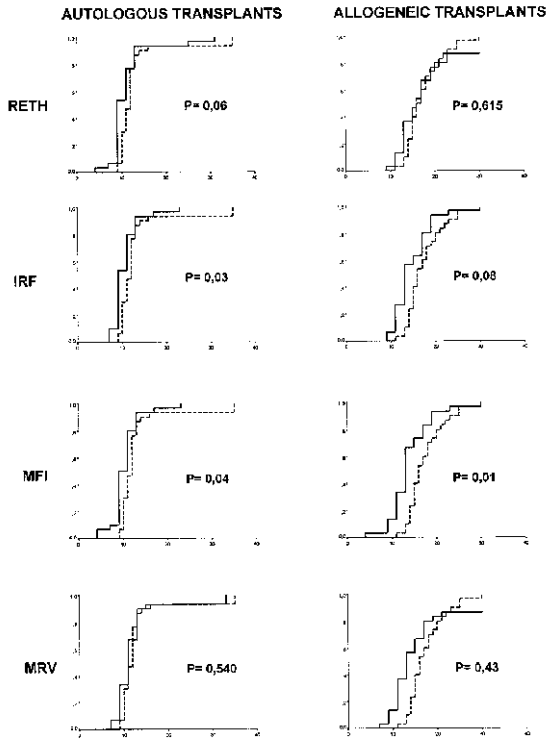

Figure 2. Kaplan-Meier plots of reticulocyte engraftment days comparing reticulocyte engraftment days and standard ANC engraftment after autologous and allogeneic transplants. The x-axis represents days after transplant and the y-axis, the cumulative frequency of achieving engraftment: the bold solid line represents reticulocyte maturation parameter engraftment and the scattered line represents standard ANC engraftment.

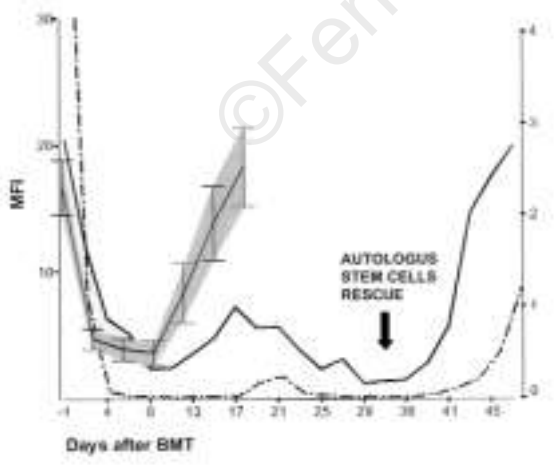

Figure 3. Schematic representation of an individual case with primary graft failure. The bold line with shaded ± SD represents the normal values for MFI for 30 allogeneic graft recipients. The single bold line represents MFI values for the patient and the scattered line displays ANC counts. The y-axis on the right represents MFI, while that on the left shows the ANC.

because most studies used different criteria to define engraftment and no study has aimed to establish a correlation model test to predict subsequent neutrophil recovery.[14,15] In this study of a large series of consecutive patients undergoing autologous or allogeneic transplants, we tested maturation parameters, RETH, IRF, MFI and MRV, as predictors of myeloid engraftment. It is noteworthy that our cut-off counts used to define erythroid engraftment were proven to be valid by finding a statistical relationship with subsequent myeloid engraftment at least for MFI and MRV. As previously reported, mean days of engraftment for RETH, IRF and MFI occurred statistically significantly earlier than engraftment defined by neutrophil count.[2,14] It is a remarkable finding that mean reticulocyte volume >110 fL, described previously in only a single case,[18] emerges as a powerful reticulocyte marker indicating the presence of larger and more immature reticulocytes 2-3 days before the neutrophil count rises. However, more useful than a simple assessment of mean differences, we succeeded in identifying MFI and MRV as predictors of myeloid engraftment in a linear correlation model for allogeneic and autologous transplants respectively. This finding could be of great importance in making clinical decisions when neutrophil engraftment seems to be delayed, and for instance when a patient requires a rescue infusion of back-up progenitor cells, as in our described patient. Nonetheless, when the day to reach an ANC of 0.1×10^9/L is taken into account, a statistically significant correlation was maintained only for MRV in the autologous setting.

The overall profile of reticulocyte maturation shows flatter response curves after a allogeneic bone marrow transplantation than after an autologous peripheral blood graft. This finding could be explained by the different sources and numbers of infused hematopoietic stem cells. Additionally during hematopoietic restoration following an allogeneic bone marrow transplant, the emergence of complete donor chimerism requires multiple immune tolerance phenomena, together with the use of methotrexate and immunosuppresive drugs to prevent acute GVHD. In conclusion, in both autologous and allogeneic transplant recipients, immature reticulocytes appear earlier than neutrophils, and there are parameters that reliably measure this fraction: RETH, IRF, MFI and MRV. Being the main statistical predictors of subsequent myeloid engraftment, MFI and MRV emerge as very useful tools in clinical practice. Patients at highest risk of graft failure, i.e. those who have received a quite low dose of infused CD34 cells, or recipients of an allograft with some degree of HLA disparity, could be closely monitored to detect any early sign of hematopoietic recovery: this monitoring is easily accomplished with automatic reticulocyte counters.

Contributions and Acknowledgments

AT had the original idea and designed the study. JS and DL participated in the conception of the study and drafted the article. JS and MAA conducted most of the statistical analyses and collected laboratory data. CV and LN

analyzed the samples. CM, AR and JC followed the patients and collected the clinical data. FM and PG supervised the study and critically revised the manuscript.

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Disclosures

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Potential implications for clinical practice

Reticulocyte parameters[19] are the earliest indicators of engraftment after hematopoietic stem cell transplantation.

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