



Assessment of hematological and immunological function during long-term follow-up after peripheral blood stem cell transplantation

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ABSTRACT

Background and Objective. Long-term hemopoietic and immunological profile after autologous peripheral blood progenitor cells transplantation (PBPC), in patients affected by hematological malignancies is largely unknown. The aim of this work was to detect the impact of high dose chemotherapy and PBPC on hemopoietic and immunological function compared to conventional chemotherapy.

Design and Methods. Patients had to fulfill the following criteria: continuous complete remission after PBPC, follow-up longer than 12 months, no chemo or radiotherapy or biological response modifiers after PBPC. Twenty-five patients were considered eligible for this analysis. Stable and complete hemopoietic reconstitution (Hb > 12 g/dL, WB > $4.0 \times 10^9/L$, ANC > $1.5 \times 10^9/L$ and Plts count > $150 \times 10^9/L$), morphological examination of peripheral blood and bone marrow, cytogenetic analysis and immunological profile were evaluated at 12 months and yearly thereafter.

Results. Immunological reconstitution showed a persistent reduction of CD4/CD8 ratio up to five years after PBPC. This reduction was related to a persistent increase of CD8⁺ lymphocytes and a constant reduction of CD4⁺ lymphocytes.

Interpretation and Conclusions. Defects observed in PBPC patients are induced by the procedure itself, by the conditioning regimen or both. The different behavior in the immune reconstitution of CD8⁺ subset after PBPC may be favored by an extrathymic origin of these cells while CD4⁺ subset recovery which is thymus-dependent is impaired after PBPC in adult population. Long-term hemopoietic reconstitution after PBPC is rapidly obtained and is stable over the years, long-term immunological function seems to be abnormal in these patients and these abnormalities are long-lasting.

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Key words: hematological reconstitution, PBPC transplantation, myelodysplasia post transplant, immune recovery

Peripheral blood progenitor cell transplantation is accepted worldwide as a therapeutic option for hematological malignancies and solid tumors.¹ This procedure was introduced

more than 10 years ago and since then it has been clear that hemopoietic recovery was fast and complete, although some concerns were raised on long hemopoietic reconstitution capacity of PBPC.^{2,3} Despite an impressive amount of scientific data on PBPC, only few data are available regarding long-term follow-up.⁴ We retrospectively analyzed data from 83 PBPC patients and isolated 25 patients who fulfilled our criteria for the evaluation of long-term hemopoietic reconstitution and function according to morphological, cytogenetical and immunological studies.

Materials and Methods

From 1988 to June 1997, 83 patients were submitted to PBPC in our Hematology Division.

Indication for PBPC was: non Hodgkin's lymphoma (NHL) in 39 patients, Hodgkin's disease (HD) in 18 patients; multiple myeloma (MM) in 17 patients acute myelogenous leukemia (AML) in 6 patients, solid tumors in 3 patients. Patients were observed after transplantation until death. Long term follow-up of hemopoietic function included bone marrow aspirate, full cell blood count (CBC), differential, reticulocyte count and lymphocytes subset. It was performed every year after PBPC. Cytogenetic analysis was performed during annual follow-up. Informed consent was obtained in all patients at the time of PBPC.

Criteria of inclusion in long-term hemopoietic reconstitution study

Patients reported in this study had to fulfill the following criteria in order to avoid interference with long-term hemopoietic reconstitution: patients had to have at least 12 months of follow-up, and had to be in continuous complete remission after PBPC. Patients receiving radiotherapy or biological response modifiers were excluded from this analysis.

Parameters for long-term hemopoietic reconstitution were considered as time to achieve Hb > 12 g/dL, WBC > $4.0 \times 10^9/L$, ANC > $1.5 \times 10^9/L$ and platelet count > $150 \times 10^9/L$ at any time after PBPC.

Demographics

Twenty-five patients fulfilled the above men-

Table 1. Patient characteristics.

No. of patients	25	
M/F	14/11	
Age (median)	40 (range 20-61)	
Disease	NHL	11
	HD	8
	MM	6
Disease status at PBPCT	CR	9
	PR	16
Conditioning regimen	BuCy2	11
	BEAM	6
Type of PBPCT	uPBPCT	20
	CD34+	5
MNCx10 ⁸ /kg reinfused (median)	6.6 (1.2-11.72)	

tioned criteria and are included in this preliminary analysis. Patient characteristics are reported in Table 1. Thirteen patients were male and 12 patients were female. Median age was 40 years (20-61). Eleven patients were affected by NHL, 6 patients were affected by MM and the remaining 8 patients by HD. All patients received standard chemotherapy at diagnosis and then received second line therapy with MiCMA (mitoxantrone 10 mg/m² day 1, carboplatinum 100 mg/m² day 1→4, cytosine arabinoside 2g/m² day 5, methylprednisolone 500 mg/m² day 1→5) or high dose cyclophosphamide (4 to 7 g/m²) G-CSF 5 mg/kg/day was administered to all patients at least 24 hours after the end of chemotherapy until completion of PBPC harvest. Patient characteristics are listed in Table 1. Disease status at PBPCT was complete remission in 9 patients and good partial remission in 16 patients. All patients were submitted to PBPCT and were conditioned with BEAM (6 pts), BuMel (8 pts) or BuCy2 (11 pts). Twenty patients were submitted to unfractionated PBPCT while 5 patients were submitted to CD34⁺ immunoselected PBPCT (Ceprate SC, Cellpro, Bothell, WA, USA). The median value of MNC reinfused was 6.6×10⁸/kg/bw (range 1.2-11.72). The fraction of CD3⁺ T lymphocytes infused with a typical PBPC collection was on average 25×10⁶/kg/bw, while the fraction of CD3⁺ T lymphocytes infused with immunoselected PBPCT was < 0.2% of MNC with a mean value of 0.18×10⁵/kg/bw (range 0.04-3.2). All patients achieved complete remission after PBPCT with a median follow-up of 24 months (14-80).

Lymphocyte phenotype

Samples were obtained during annual follow-up. Short-term immunological reconstitution has been already previously reported.⁵ Briefly, T-cell immune reconstitution during the first year was markedly depressed in patients receiving immunoselected

CD34⁺ progenitors as compared to patients transplanted with unfractionated PBPC (manuscript in preparation). Double-labeling experiments were performed on EDTA anticoagulated blood samples; aliquots of 100 mL were incubated for 30 minutes at 4°C with FITC or PE conjugated mAb: CD45, CD4, CD8, CD16, HLA-DR, CD56. Isotype matched control antibodies were used as controls. Erythrocytes were lysed by adding 3 mL of NH₄Cl/EDTA for 10 minutes at room temperature; cells were then washed in PBS-EDTA and kept on ice until FACS analysis (FACScan, Becton Dickinson, USA).

Analysis of peripheral blood counts

Full blood counts were carried out monthly during the first year after PBPCT and every 3 months thereafter. Hemograms and WBC differential counting were obtained with the Bayer Technicon H3 system. reticulocyte counting and percentage of immature fraction reticulocyte (IFR) resulting from the addition of highly fluorescent reticulocyte (HFR) and medium fluorescent reticulocyte (MFR) values were estimated using a Sysmex R1000™ (Merck Clevenot, France)(TOA Medical Electronic Co, Kobe, Japan), a fully automated flow cytometric reticulocyte counter. Bone marrow aspirate with morphological study to evaluate the presence of dysplasia in at least one hemopoietic lineage and cytogenetic studies requiring the presence of at least 20 metaphases were obtained during annual follow-up in all pts. Myelodysplasia was defined by FAB criteria.⁶

Control group

Ten patients affected by NHL (6 patients) and HO (4 patients) submitted to standard chemotherapy (AB VD, MOPP, F-MACHOP, PROMace Cytabom) and second-line treatment with MiCMA or other chemotherapeutic protocol, not submitted to PBPCT and comparable for age, status of disease stage, and number of previous treatment were used as control group. The median follow-up was 15 months (7-54 months).

Statistical methods

Correlations were studied using Spearman rank analysis. Comparisons were performed with Wilcoxon W test for paired determinations, defining the criterion for statistical significance as p<0.05.

Results

Twenty out of 25 patients (80%) achieved a stable and complete hemopoietic reconstitution as: Hb > 12 g/dL, WBC > 4.0×10⁹/L, ANC > 1.5×10⁹/L and platelet count > 150×10⁹/L at a median of 91 days (0-575), 22 days (11-285), 15 days (12-219), 76.5 days (11-408) after PBPCT respectively. Five

patients (20%) lack 1 out of 3 criteria for long-term hemopoietic reconstitution: 3 patients did not achieve Hb >12 g/dL at maximum follow-up of 2190 days and 2 patients did not achieve a platelet count >150×10⁹/L at maximum follow-up of 900 days. Nevertheless, all of them achieved a stable Hb value >10 g/dL at day +38, +365 and 1493 and a platelet count >100×10⁹/L at day +14 and +91, respectively (Table 2).

Immature reticulocyte fraction was within the normal range with a median value of 16.2% (range, 8.3-29) and reticulocyte count was 1.56% (range, 0.76-2.43).

Immunological recovery of transplanted patients was characterized by a persistent reduction of the CD4/CD8 ratio with a median value of 0.5 (range, 0.2-1.1), a reduction of CD4⁺ lymphocytes with a median value of 0.472×10⁹/L (range, 0.176-0.9) and an increase of the CD8 lymphocytes with a median value of 0.933×10⁹/L (range, 0.390-4.7). We also observed an increase of NK population with a median value of 0.260×10⁹/L (range, 0.114-1.2) and activated T-cells coexpressing HLA-DR and CD3 antigens with a median value of 0.258×10³/L (range, 0.037-2.42). CD3⁺ lymphocytes were within the normal range with a median value of 1330×10³/L, while a small increase of CD19⁺ cells was observed with a median value of 0.415×10³/L (range, 0.137-2.77). Results are summarized in Table 3.

Assuming an arbitrary cut-off at 24 months after PBPC (our median time of follow-up) we observed a trend toward normalization which was significant for CD4/CD8 ratio ($p=0.03$), CD4⁺ value ($p=0.0002$) and CD3⁺ value ($p=0.05$) as reported in Figure 1. No statistically significant results were obtained for CD8⁺, NK, CD3/HLA-DR and CD19⁺ value before or after 24 months of follow-up. No difference was found in terms of T-cell reconstitution beyond 1 year of follow-up between patients submitted to unfractionated PBPC or immunoselected CD34⁺ progenitors transplantation while short-term T cell reconstitution was delayed in patients undergoing immunoselected CD34⁺ PBPC. Although the number of CD3⁺ T lymphocytes infused with the inoculum in the 2 groups is significantly different the number of patients is too small to draw any final conclusion.

Immunological recovery was not influenced by age, underlying disease, conditioning regimen or type of PBPC.

The morphological examination of bone marrow revealed no consistent features of MDS or acute leukemia, while it showed minor dysplastic changes including Jolly bodies, karyorrhexis and nuclear/cytoplasmic asynchronism in erythroid lineage in 4 patients, nuclear hypolobularity, hypogranularity in myeloid lineage in 2 patients and the occurrence of binucleated megakaryocytes and micro-

Table 2. Long-term hemopoietic reconstitution.

Hematological parameters	No. of pts. (%)	Median day (range)
Hb > 12 g/dL	22 (88)	91 (0-575)
WBC > 4×10 ⁹ /L	25 (100)	22 (11-285)
ANC > 1.5×10 ⁹ /L	25 (100)	15 (12-219)
Plt > 150×10 ⁹ /L	23 (92)	76.5 (11-408)

Table 3. Immunological reconstitution.

	Median value (range)	Normal value
CD4×10 ⁹ /L	0.472 (0.176-0.9)	(0.670-0.950)
CD8×10 ⁹ /L	0.933 (0.390-4.7)	(0.505-0.695)
CD4/CD8	0.5 (0.2-1.1)	(1.1-1.8)
CD16/56 ⁺ ×10 ⁹ /L	0.260 (0.114-1.2)	(0.070-0.190)
CD3/DR ⁺ ×10 ⁹ /L	0.258 (0.037-2.42)	(0.04-0.155)
CD3×10 ⁹ /L	1.33 (0.624-5.45)	(1.185-1.540)
CD19×10 ⁹ /L	0.415 (0.137-2.77)	(0.160-0.290)

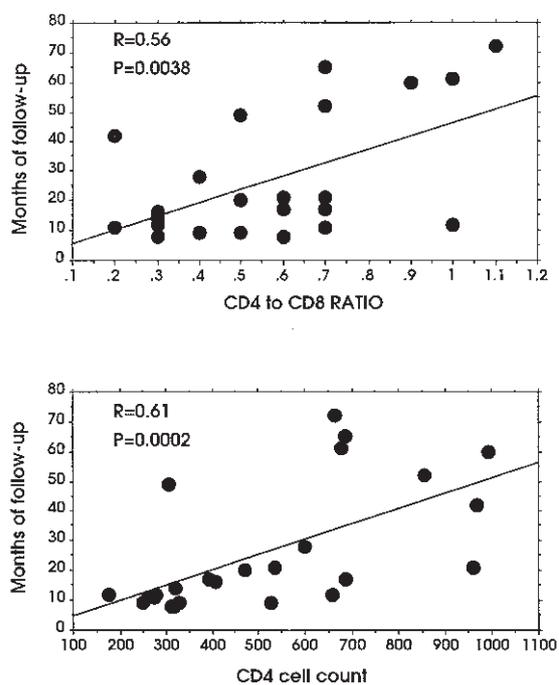


Figure 1. Correlation between follow-up (months), CD4/CD8 ratio and CD4 absolute cell count.

megakaryocytes in 2 patients. All changes observed were transient and not consistent with diagnosis of myelodysplastic syndrome.

No cytogenetic abnormalities were observed at any time during this follow-up.

The control group submitted only to chemotherapy showed a substantial difference concerning immunological recovery compared to PBPC patients. In fact, a CD4/CD8 ratio at the same median follow-up of the transplanted group was 0.93 (range, 0.83-1.7) ($p < 0.0001$), with a median value of CD4⁺ $0.650 \times 10^9/L$ (range, 0.230-1.32) ($p = 0.03$) and a median value of CD8⁺ $0.692 \times 10^9/L$ (range, 0.254-1.43) ($p = ns$).

In the control group CD3/HLA-DR⁺, CD3⁺, and CD19⁺ subpopulations were within the normal range while a small increase was observed in NK subpopulations with a median value of $0.096 \times 10^9/L$ (range, 0.037-0.77).

Discussion

PBPC is a worldwide accepted anticancer strategy in hematological malignancies and solid tumors. This approach is very appealing due to the low toxicity encountered caused by a very fast recovery of hematological parameters. Since PBPC was first introduced in the late '80s, follow-up time for patients submitted to PBPC has now elapsed long enough to evaluate the incidence of late effects on long-term hemopoietic reconstitution.^{2,3} In this paper we investigated the long-term hemopoietic function in a selected group of patients submitted to PBPC observed for at least 12 months. Patients were selected on the basis of very select criteria in order to avoid interference with the hemopoietic function arising from disease recurrence or from further chemotherapy including biological response modifiers or cytokines.

Our results showed a very high percentage of complete and self-sustaining long-term hemopoietic reconstitution with 80% of patients achieving a normal CBC all reticulocyte parameters. The morphological study of bone marrow peripheral blood smear conducted regularly during follow-up showed, in a minority of patients, only single lineage abnormalities which were not sufficient for the diagnosis of myelodysplastic syndrome according to FAB criteria.^{7,8} No cytogenetic abnormalities were observed. Unexpectedly, we found significant abnormalities in long-term immunological reconstitution in virtually all patients. These abnormalities were primarily characterized by a reduction of CD4⁺ lymphocytes and by an increase of CD8⁺ lymphocytes. As a result of this imbalance, a persistent reduction of CD4/CD8 ratio was observed. During further follow-up, a significant trend toward normalization of CD4⁺ lymphocytes subset and CD4/CD8 ratio was observed after 24 months.

Patients submitted to PBPC also showed a persistent increase of NK cells throughout the follow-up. There was no influence of age, conditioning regimen, type of PB PCT or disease on CD4⁺ lymphocytes recovery while CD8⁺ lymphocytes fraction was particularly increased in patients over the age of 40. The different behavior in the immune reconstitution of CD8⁺ subset after PBPC as recently reported,⁹ may be favored by an extrathymic origin of these cells while CD4⁺ subset recovery which is thymus-dependent is impaired after PBPC as expected in this adult population.¹⁰

A different regenerative pathway of CD4⁺ and CD8⁺ lymphocytes after chemotherapy has been recently suggested by Mackall *et al.* In fact, in their study, CD4⁺ recovery was poor and clearly age and thymus dependent while CD8⁺ recovery was fast and age and thymus independent.¹¹ Moreover, the coexpression of CD28 antigen on CD8⁺ lymphocytes was also evaluated to discriminate the thymic influence on CD8⁺ maturation. These observations in humans have been recently confirmed in murine model after marrow transplantation.¹²

Despite the number of CD3⁺ T lymphocytes infused with the inoculum in patients undergoing immunoselected CD34⁺ PBPC is significantly different, T cell reconstitution beyond 1 year does seem to be affected but the number of patients is too small to draw any firm conclusion. Short-term T cell reconstitution is under evaluation in a larger group of patients in order to explore whether the number of infused T cells has a significant impact on the pattern of immune reconstitution.

Our results differ from other reports, in which a rapid normalization of CD4⁺ subset and CD4/CD8 ratio was achieved after PBPC.^{13,14} In these studies patients were observed after a much shorter follow-up and thus results are difficult to compare. In fact, patients in this study were considered eligible only if a minimum of 1 year had elapsed from PBPC. Furthermore, only patients considered in remission were included in the study. Interestingly, the influence of previous chemotherapy history was ruled out in this study by the observation during follow up of a control group not submitted to PBPC. No abnormalities were found in the lymphocytes subset compared to patients submitted to PBPC, thus leading to speculation that the defects observed in PBPC patients were induced by the procedure itself, by the conditioning regimen or both. Whether these long-lasting modifications of fine immunological tuning are relevant to disease control after PBPC or to the development of late effects (myelodysplasia, infectious complications) will be the challenge for the future. In the meanwhile, long-term follow-up is hoped for patients submitted to PBPC in order to detect late sequelae of this widespread anticancer treatment.

Contributions and Acknowledgments

LL collaborated in the study design, data handling and was the principal clinician involved, SS was responsible for the conception of the study, its design, ethical approval, interpretation of data and direct supervision, PS was responsible for interpretation of data and wrote the paper with LL and SS, SR and CR were responsible for all the immunological data, RS was responsible of PBPC collections, GD was responsible for all the morphological study, GL revised the manuscript and gave final approval.

The order in which authors name appear is the following: LL, SS and PS wrote the manuscript, collected the data and made the analysis of the results and took care of transplanted patients, SR provided the immunological data and collaborated to statistical analysis, RS provided data on leukapheretic procedures, GD, CR and GL are responsible for reviewing the data and the manuscript.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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