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*Original Article*

# Detection of minimal residual disease in hematopoietic progenitor cell harvests: lack of predictive value of peripheral blood and bone marrow analysis in mantle cell and indolent lymphoma

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**Abstract:** Elimination of neoplastic cells from peripheral blood progenitor cells (PBPCs) is an important issue in transplantation-based high-dose chemotherapy in non Hodgkin's lymphoma (NHL). The capacity to reliably assess the presence of residual lymphoma cells in PBPCs is mandatory in designing this type of protocols. Polymerase chain reaction (PCR) amplification of molecular rearrangements is widely used to detect minimal residual disease (MRD) in NHL patients. Although concordant data can be obtained in most of the cases from peripheral blood (PB) and bone marrow (BM) at diagnosis, the relationship between these two compartments and the role of their analysis in predicting the molecular status of PBPCs is still an open issue. Here we report data about MRD analysis in BM, PB and PBPCs in a series of mantle cell and indolent NHL patients who underwent high-dose chemotherapy: discordant results were obtained comparing PB, BM and PBPC molecular data. In addition, differences were noted among these results if molecular analysis was performed using well-known rearrangements (i.e., bcl-1/IgH and bcl-2/IgH) or patient specific oligonucleotides. We conclude that neither BM nor PB are reliable in predicting the molecular status of PBPCs and that caution must be adopted in interpreting molecular data obtained using patient specific oligonucleotides.

**Keywords:** Minimal residual disease, peripheral blood, bone marrow, peripheral blood progenitor cells

## Introduction

Malignant lymphoma is the most common hematologic malignancy encountered in the Western world [1]. Although progress has been made in the treatment of advanced stage lymphomas, the majority of patients ultimately relapse. In this context, myeloablative therapy followed by autologous hematopoietic progenitor cell rescue has an established role in the management of the disease [2]. In clinical practice, autologous transplantation of PBPCs is preferred to autologous bone marrow transplantation (ABMT) because it produces a significant improvement in the therapeutic index of high-dose chemotherapy, reducing iatrogenic toxicity [3]. The role for purging in ABMT for patients with NHL was suggested by studies that strongly indicated the contribution of residual lymphoma cells to relapse [4]. In this regard, clinical programs have been developed to assess the usefulness of in vitro and in vivo purging procedures also when using PBPCs, and lately in vivo purging has been described as a promising therapeutic strategy [5].

BM and PB involvement by lymphoma has traditionally been detected by morphologic and immunophenotypic analysis. In the last few years, molecular biology techniques have been used for detection of MRD with the aim to provide prognostic indications. In this context, studies

have been performed to find out whether BM and PB are equivalent as tissue sources to detect residual disease [6]. The results have clearly shown that detection of residual lymphoma cells in BM after therapy is associated with decrease in disease-free survival [7]. Thus, it has been concluded that BM is more informative than PB in detecting MRD both at the time and after ABMT [6].

With the aim to evaluate the usefulness of PB and BM PCR analysis to predict the molecular status of PBPCs and thus prospectively guide their harvest, we recently analyzed these three hemopoietic compartments in a series of 30 NHL patients. Our data show a lack of absolute concordance in the detection of residual lymphoma cells in PB, BM and PBPCs, assessed by PCR using well known chromosomal translocation (e.g. bcl-2/IgH and bcl-1/IgH) or allele-specific oriented oligonucleotides based on patient CDRIII sequences. Although preliminary, our results seem to indicate that caution must be adopted in assuming that molecular data obtained from PB or BM are equivalent to those obtained from PBPCs and that the type of the molecular marker used during the PCR analysis has to be taken into account evaluating the data.

## Patients and methods

*Patients and treatment plan*

Between December 1996 and March 2001, 30 consecutive patients with mantle cell and indolent NHL received high-dose sequential chemotherapy with autologous PBPC support and rituximab (R-HDS). Eligibility criteria included written informed consent; age ≤60 years; absence of severe organ disfunctions not due to tumor; no prior viral infections (HBV, HCV, HIV); a histologically confirmed diagnosis of mantle cell lymphoma or follicular lymphoma, either refractory to or relapsed within one year following first -line polychemotherapy, and requiring treatment; expression of CD20 by lymphoma cells; and availability of a molecular probe for PCR amplification of DNA.

All patients received chemotherapy based on the treatement plan as already described in detail [5]. Briefly, after an initial standard-dose phase consisting of 2 to 3 cycles of either doxorubicin- or cisplatin-containing chemotherapy, all patients were assigned to receive a rituximab-supplemented 4-step high-dose sequence including intravenous administration of: high-dose cyclophosphamide (7g/$m^2$), high-dose cytarabine (2 g/$m^2$ every 12 hours for 6 consecutive days), high-dose melphalan (180 mg/$m^2$), and high-dose mitoxantrone plus melphalan (60 and 180 mg/$m^2$, respectively). Rituximab was infused intravenously for a total of 6 doses, twice after cyclophosphamide, twice after cytarabine, and twice after the final myeloablative step.

*Timing, harvesting, and processing of PBPC*

Timing and number of collections was prospectively guided by real-time assessment of circulating PBPC counts [8], as well as by results of overnight PCR analysis on a sample of harvested cells. PBPCs were assessed by counting total $CD34^+$ cells by direct immunofluorescence flow cytometry (FACS) with the phycoerythrin-conjugated HPCA-2 CD34 antibody (Becton Dickinson, San Jose, CA) as previously described [9].

PBPC harvests were performed during growth-factor expanded mobilization of progenitor cells occurring after either high-dose cyclophosphamide, or high-dose cytarabine, or both. Mononuclear cells were collected by use of an automated leukapheresis system (SPECTRA AutoPBSC, COBE, Lakewood, CO), as previously described [10].

*BM, PB and PBPC samples preparation*

BM and PB samples were obtained from all the 30 patients at the time of the initial evaluation and on the day of PBPC harvest after high-dose cyclophosphamide. PBPC samples were collected at the end of each harvest. Samples preparation was carried out by alkaline lysis of red cells by ACK lysing buffer [11] (ACK, BioWhittaker, Walkersville, MD, U.S.A.) and DNA extraction performed using the QIAamp DNA blood extraction kit (Qiagen, Valencia, CA, U.S.A.).

*PCR amplification*

Molecular monitoring of MRD was performed by analyzing DNA samples from PB, BM and PBPCs with use of either nested PCR amplification of bcl-2/IgH translocation or semi-nested amplifi-

cation of bcl-1/IgH and clonal rearrangement of IgH genes, essentially as already described [12, 13]. Briefly, for bcl-2/IgH translocation, amplification of major (MBR) and minor (mcr) was performed using oligonucleotide primers originally designed by Gribben [14], while bcl-1/IgH translocation was detected using the primers already described by Rimokh [15]. Tumor VDJ was amplified using consensus VH.D and JH.D primers already designed by Deane [16] (FR1 primers). Amplified DNA was directy sequenced using VH.D and JH.D primers and VH-, D-, JH- regions and N-inserts were identified by BLAST. The CDRIII region was identified as the junction of these three regions including the N-inserts. 20mer antisense allele-specific oligonucleotides (ASO) primers were designed from the CDRIII regions including N-insert. DNA from the patient samples was amplified by semi-nested PCR: the first amplification used the relevant VH.D family and JH.D consensus primers, while the second amplification was performed with the same VH.D primer and the designed ASO antisense primer. Amplified DNAs were analyzed by electrophoresis on 1.5% agarose gels containing ethidium bromide and visualized by UV light.

*Flow cytometry*

Cells from PB were stained for 30 minutes on ice with combinations of saturating amounts of fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated monoclonal antibodies. FITC- or PE-conjugated antibodies against CD5, CD19 and CD23 were purchased from Becton Dickinson (San Jose, CA, U.S.A.). Each fluorescence analysis included appropriate PE- and FITC-conjugated negative isotype controls. The percentage of positive cells was determined by subtracting the percentage of fluorescent cells in the control from the percentage of cells positively stained with the appropriate antibody. The cells were analyzed by using a FACScan laser flow cytometry system (Becton Dickinson) equipped with a Macintosh Power-Mac G3 personal computer (Apple Computer, Cupertino, CA, U.S.A.) and Cell Quest (Becton Dickinson) software.

*Sensitivity and specificity*

The sensitivity of detection of MRD was assessed by PCR amplification of 10-fold serial dilutions of tumor cell DNA into normal donor PB DNA. The limit of detection of MRD was reproducible at the level of $10^{-6}$ for the bcl-2/IgH translocation and at $10^{-5}$ for the other two rearrangements. A patient-specific positive control was included in all PCR reaction; negative controls containing 1) water instead of DNA and 2) polyclonal DNAs were added. To further test sensitivity, every and each amplification contained a weak-positive, patient-specific control consisting of a $10^{-5}$ to $10^{-6}$ dilution of tumor cell DNA into normal donor PB DNA; the reaction was thus considered as adequately sensitive only if this latter sample gave a positive signal. All PCR reactions were performed in duplicate, in at least two independent experiments.

Specificity was determined by using the diagnostic sample as positive control and BM samples from three other patients as negative controls.

## Results

*Patient characteristics*

The main characteristics of the 30 NHL patients analyzed are listed in **Table 1**. Histologic findings confirmed the diagnosis of follicular or mantle cell lymphoma in all the patients; the diagnosis of this latter group was confirmed by expression of CD5 and lack of co-expression of CD23 on the tumor cells. Bcl-2/IgH rearrangement was detected in 10 out of 21 follicular lymphoma patients, and bcl-1/IgH rearrangement was present in 5 out of 9 mantle cell lymphoma cases. The majority of patients were diagnosed as stage IV disease.

**Table 1.** Patient characteristics

| Characteristic | # |
|---|---|
| Histologic findings | |
| Follicular, grade 1 | 15 |
| Follicular, grade 2 | 2 |
| Follicular, grade 3 | 4 |
| Mantle cell disease | 9 |
| Molecular rearrangement | |
| Bcl-2/IgH | 10 |
| Bcl-1/IgH | 5 |
| IgH | 15 |
| Stage | |
| III | 7 |
| IV | 23 |

*Detection of lymphoma cells in BM and PB at diagnosis*

Routine morphological assessment and immunohistochemical staining of BM sections with

**Table 2.** Histological and molecular analysis of BM and PB at diagnosis

| Pt | BM hist | BM PCR | PB PCR | probe |
|---|---|---|---|---|
| 1 | + | + | + | |
| 2 | + | + | + | |
| 3 | + | + | + | |
| 4 | + | + | + | |
| 5 | - | + | + | bcl2 |
| 6 | + | + | + | |
| 7 | + | + | + | |
| 8 | + | + | + | |
| 9 | + | + | + | |
| 10 | + | + | + | |
| 11 | - | + | + | |
| 12 | + | + | + | |
| 13 | - | + | + | |
| 14 | - | + | + | |
| 15 | + | + | + | |
| 16 | - | - | - | IGH |
| 17 | + | + | + | |
| 18 | + | + | + | |
| 19 | - | + | + | |
| 20 | - | - | - | |
| 21 | - | - | - | |
| 22 | + | + | + | |
| 23 | + | + | + | |
| 24 | + | + | + | bcl1 |
| 25 | + | + | + | |
| 26 | + | + | + | |
| 27 | - | + | + | |
| 28 | + | + | + | |
| 29 | + | + | + | IGH |
| 30 | - | + | + | |

Neg: -; Pos: +

anti-CD3 and anti-CD20 antibodies were performed in all 30 cases at diagnosis. The evaluation of BM by these techniques allowed to identify lymphoma cells in 20 out of 30 patients (66%). Conversely, molecular analysis detected lymphomatous involvement in 27 samples analyzed (90%) (**Table 2**).

PB was also evaluated: PCR showed lymphomatous involvement in 27 out of 30 patients (90%). In addition, in mantle cell lymphoma patients flow cytometry allowed to detect circulating lymphoma cells (CD5+/CD19+/CD23-) in 7 out of 9 patients (78%) (**Figure 1A**), while in the same group, molecular analysis gave evidence of a monoclonal population in 9 out of 9 cases evaluated (**Figure 1B**).

*Comparison of PBPC harvest, PB and BM PCR analysis*

To assess the role of BM and PB data obtained by PCR analysis in predicting the molecular status of PBPCs, samples of the former compartments collected at the time of PBPC harvest were analyzed together with the latter. As shown in **Table 3**, in 4 out of 13 patients (30%) whose BM scored positive at PCR analysis, PBPCs were found to be negative. In the remaining 17 patients, PCR analysis was unable to detect lymphoma cells in BM: however, contaminating lymphoma cells were detected by PCR in the PBPCs of 5 of these patients (29%).

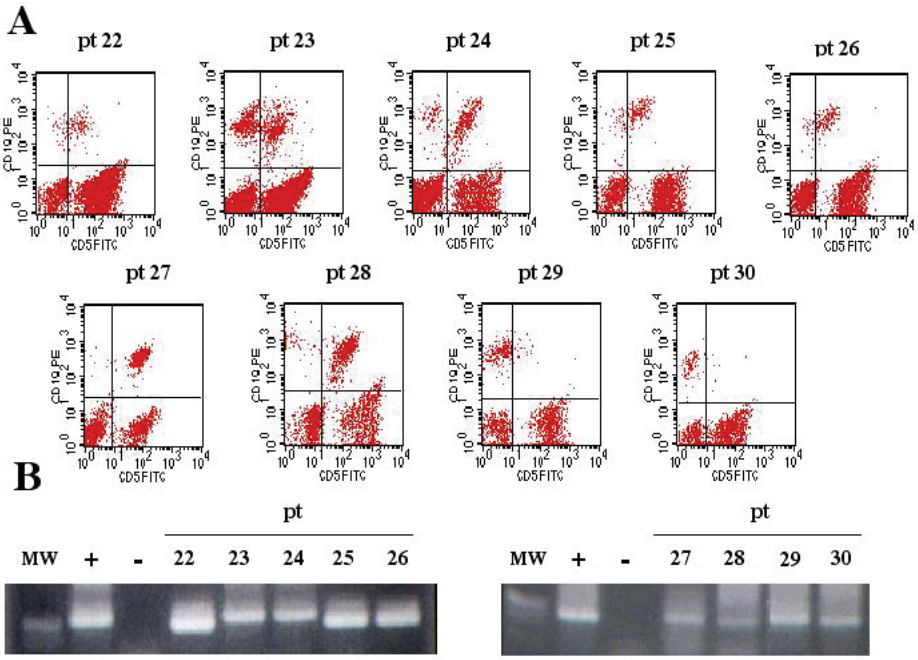


**Figure 1.** FACS and PCR analysis of PB in mantle cell lymphoma patients at diagnosis. A: flow cytometric characterization of PB; dot plots show the CD5/CD19 distribution of B-lymphocytes; B: PCR analysis of PB; pts 22 – 26 were analyzed by bcl-1/IgH rearrangement, pts 27 – 30 were analyzed by CDR3-based oligonucleotides; MW, molecular weight standards; (+), positive control; (-), no DNA. Samples were analyzed on 1.5% agarose gel and stained with ethidium-bromide.

**Table 3.** Molecular analysis of BM, PB and PBPC

| Pt | BM | PBPC | PB | probe |
|---|---|---|---|---|
| 1 | + | - | - | |
| 2 | + | + | - | |
| 3 | + | - | - | |
| 4 | + | - | - | |
| 5 | - | - | - | bcl2 |
| 6 | + | + | - | |
| 7 | + | + | + | |
| 8 | - | + | - | |
| 9 | + | + | - | |
| 10 | + | + | + | |
| 11 | - | - | - | |
| 12 | + | + | + | |
| 13 | - | - | - | |
| 14 | - | - | - | |
| 15 | - | + | - | |
| 16 | - | - | - | IGH |
| 17 | - | + | - | |
| 18 | - | - | - | |
| 19 | - | - | - | |
| 20 | - | - | - | |
| 21 | - | - | - | |
| 22 | - | + | - | |
| 23 | - | - | - | |
| 24 | + | + | + | bcl1 |
| 25 | + | + | + | |
| 26 | + | - | - | |
| 27 | - | - | - | |
| 28 | - | - | - | |
| 29 | + | + | + | IGH |
| 30 | - | + | + | |

Neg: -; Pos: +

Molecular analysis of PB was performed on triplicate samples from each patient, immediately before PBPC harvest. As shown in **Table 3**, a positive PCR signal was invariably detected in PBPC samples of the 7 patients whose PB scored positive at PCR analysis. In 7 of the remaining 23 patients (30%) whose PB scored negative, PBPCs resulted positive at PCR analysis.

*PB immunophenotyping: comparison with molecular data*

The predictive value of PB immunophenotyping was assessed at the time of PBPC harvest in the subgroup of mantle NHL patients by means of flow cytometry after staining with anti-CD5, anti-CD19 and anti-CD23 monoclonal antibodies. The results of FACS and PCR analysis were compared (**Table 4**): immunophenotyping was negative for presence of CD5+/CD19+ cells in 7 out of 9 patients (**Figure 2A**); however, in 2 of these patients (28%), PCR analysis was able to detect a signal corresponding to the presence of lymphoma cells (**Figure 2B**). In the remaining 2 cases, FACS analysis detected CD5+/CD19+ cells and PCR scored positive.

**Table 4.** PB immunophenotyping vs. PCR

| Pt | FACS | PCR |
|---|---|---|
| 22 | - | - |
| 23 | - | - |
| 24 | + | + |
| 25 | - | + |
| 26 | - | - |
| 27 | - | - |
| 28 | - | - |
| 29 | - | + |
| 30 | + | + |

Neg: -; Pos: +

## Discussion

Residual neoplastic lymphocytes in hematopoietic progenitor cells reinfused in B-NHL patients during autologous transplantation may be an important cause of relapse. It has already been demonstrated by others that tumor cells contaminating BM harvest used in autologous transplantation can contribute to disease recurrence [4, 17, 18], and some recent reports indicate that this may be true also when using PBPCs [19].

Although BM and PB analysis at the end of the treatment has a well defined role in predicting lymphoma relapse [6], it is still a matter of debate whether the same tissues are adequate for predicting MRD in PBPCs. Some recent papers have already given interesting insights about this isssue, although for different types of NHLs [20] or after different therapeutic strategies [21]. Thus, additional indications about this issue may be helpful to prospectively guide PBPC harvesting procedures, in particular when ex-vivo purging strategies have to be considered.

In the present study, we tried to qualitatively evaluate if data obtained from BM and PB are predictive for the molecular status of PBPCs. To this aim, in a series of NHL patients who underwent high-dose chemotherapy, we analyzed PB and BM samples obtained at the time of PBPC

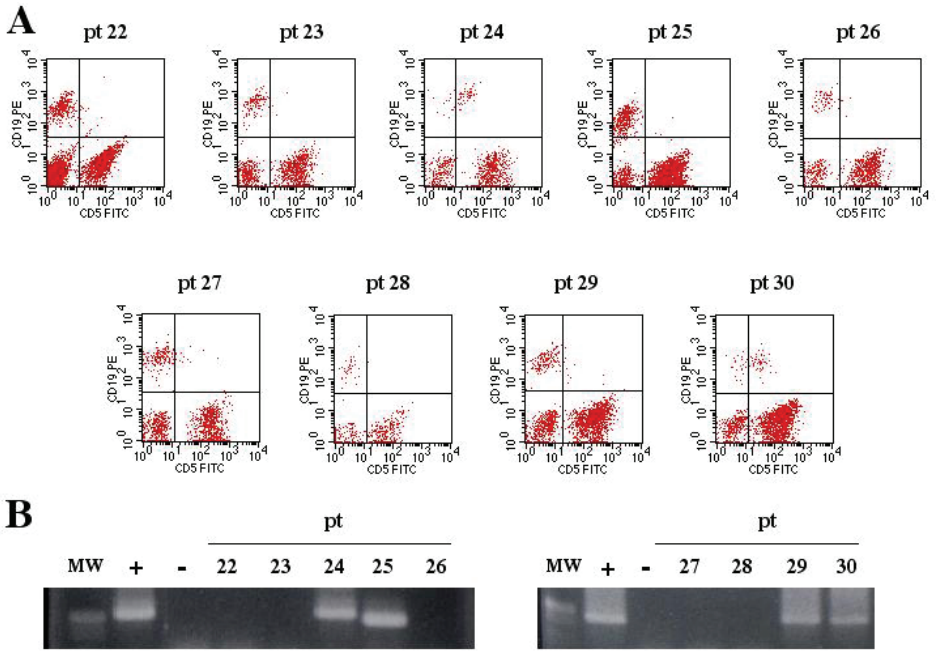


**Figure 2.** FACS and PCR analysis of PB in mantle cell lymphoma patients at the time of PBPC harvest. A: flow cytometric characterization of PB; dot plots show the CD5/CD19 distribution of B-lymphocytes; B: PCR analysis of PB; pts 22 – 26 were analyzed by bcl-1/IgH rearrangement, pts 27 – 30 were analyzed by CDR3-based oligonucleotides; MW, molecular weight standards; (+), positive control; (-), no DNA. Samples were analyzed on 1.5% agarose gel and stained with ethidium-bromide.

collection: among patients who had a PCR-negative BM, 29% still had positive PBPCs. Discordance was also evident comparing data obtained from PB and PBPCs: PCR-positive PBPCs were harvested in 30% of PCR-negative PB patients. In addition, the comparison of the results obtained by immunophenotyping and by PCR, showed that 2 out of 7 patients (28%) who scored negative at FACS analysis were found to have positive PB and PBPCs when analyzed by PCR. These findings question the value of PB immunophenotyping as a predictive assay for the evaluation of PBPCs.

PCR amplification is widely being used to detect lymphoma cells in BM and PB. In this respect, our results confirm that at diagnosis, when tumor burden is relevant, there is a concordance between BM and PB histological and molecular data, as reported by other groups [22]. However, a note of caution has to be made about the interpretation of these data when chemotherapy is administered: in this case, there is a decrease in tumor burden, blood-marrow barrier is altered [23] and cytokines probably modify the regulation of cell adhesion molecules [24, 25]. In this situation PCR sensitivity becomes critical: in fact, depending on the marker used, the percentage of positive BM samples collected after chemotherapy, at the time of PBPC harvest, could range from 66% (bcl-2/IgH or bcl-1/IgH) to 16% (patient specific oligonucleotide). These data are in accordance with those obtained by other groups [26].

In the case of a PCR-negative BM, harvesting PCR-positive PBPCs may have different explanations such as i)an enrichment in neoplastic lymphocytes during PBPC harvest and ii)a negativity of BM samples for failure to aspirate the diagnostic cells due to residual reticulin fibrosis often associated with a lymphomatous infiltrate. However, it cannot be completely excluded that the detection of MRD in PBPCs may be due to the expansion and release in the bloodstream of the lymphoma cell population as a result of hematopoietic growth factor stimulation and their action on the growth rate and adhesion receptors of lymphoma cells as already suggested by other authors [24, 25].

An explanation for a PCR-negative PB patient whose harvest scores PCR-positive may be the administration of rituximab, sufficient to clear the former but not the latter from neoplastic cells. In this respect, it is thus not surprising that nearly 50% of these cases have a positive BM, since the clearance of B-cells might be more rapid in PB than in BM.

Finally, an interesting issue that remains to be solved is about the cases with PCR-positive PBPCs who score negative both at BM and PB level (cases 8, 15, 17, 22): this could be due to

recruitment of neoplastic cells in PBPCs from other sites (i.e. spleen, liver) by unknown mechanism, in a way similar to intra-apheresis CD34+ cell recruitment described by some authors [27].

Taken together our data suggest that, in patients with indolent and mantle cell NHL, bcl-2/IgH and bcl-1/IgH PCR is superior to PCR based on CDIII sequences in terms of sensitivity, and that this technique has to be applied to PBPCs, and not to PB or BM, to assess neoplastic contamination of the harvests. As the number of patients is small, final conclusions about the clinical importance of our findings awaits further study. In addition, in the near future, quantitative rather than qualitative assays for the evaluation of MRD in PBPCs are desirable to evaluate and optimize chemo-immunotherapy protocols and PBPC mobilization regimens.

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