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Unmanipulated peripheral blood stem cell autograft in chronic lymphocytic leukemia: clinical findings and biological monitoring

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ABSTRACT

Background and Objectives. **To investigate the feasibility of peripheral blood stem cell (PBSC) transplantation in patients with high-risk chronic lymphocytic leukemia (CLL) in remission after fludarabine therapy, the clinical impact of minimal residual disease (MRD) monitoring and the immunologic reconstitution after transplantation.**

Design and Methods**. Twenty CLL patients, in clinical complete remission (CR) after fludarabine, were offered an unmanipulated PBSC transplant and were longitudinally monitored for MRD and immunologic reconstitution.**

Results. **Due to unsatisfactory PBSC collection, 4 patients received bone marrow cells. All patients engrafted. Two patients died, one due to infection and one because of another neoplasia. Thirteen patients are at present in clinical CR after a median follow-up of 17 months and 18 patients are alive with a survival probability of 0.87 (±0.04) at 52 months after transplant. Fifteen patients had a molecular remission. Three of them showed a molecular relapse 16-28 months after autograft, followed by a clinical relapse 10-16 months later. Three of the four patients who showed persistent rearrangement could be re-evaluated over time and showed an immunologic relapse 11-26 months after transplant; two of these had a clinical relapse 12 and 7 months later. A marked and persistent impairment of both the B- and T-immunologic compartments was recorded in the longitudinal follow-up.**

Interpretation and Conclusions**. Unmanipulated PBSC autograft is a feasible procedure that produces prolonged molecular remissions in high-risk CLL patients. Persistence or reappearance of a molecular signal after engraftment is predictive of subsequent immunologic and clinical CLL recurrence. The long-lasting impairment of the host immune repertoire after fludarabine followed by autograft has to be taken into account in the patients' management.**
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Key words: chronic lymphocytic leukemia, fludarabine, PBSC autograft

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Chronic lymphocytic leukemia (CLL) has a heterogeneous often indolent natural history.[1,2] The annual mortality rate is about 8%, median survival is nearly 6 years and 20% of patients survive more than 10 years. While the overall median age at diagnosis is 65 years, approximately 30% of CLL patients are younger than 60 years at the time of presentation.[1-3] CLL is incurable by conventional treatment modalities and in the presence of adverse prognostic factors survival is shorter than 3 years. This prognostic likelihood is not acceptable for younger patients,[3] for whom new treatment strategies are required in an attempt to provide long-lasting remissions and, possibly, to eradicate the disease. While the management of patients with CLL remained largely unmodified over the last three decades, recently new therapeutic approaches have become a realistic option. The purine analog fludarabine has proven capable of inducing better quality remissions and in relatively young patients with CLL autologous transplant represents a feasible and promising treatment.[2-6] High-dose therapy followed by infusion of autologous progenitor cells can induce complete remissions (CR) in a proportion of patients, with evidence of molecular disappearance of the disease in some cases.[5-19] In the majority of studies, bone marrow (BM) has been employed as the source of stem cells.[8-11] However, the greater peripheral blood progenitor cell content and the results obtained in other hematologic malignancies have induced several investigators to consider the possibility of using peripheral blood stem cells (PBSC) also for autografting CLL patients.[11-19]

Although the impact of an autograft on the duration of disease-free survival (DFS) and overall survival (OS) remains to be established, there are also many other issues which need to be adequately addressed. In particular, the presence and role of possible residual leukemic B-cells in the recurrence of CLL after autograft and the methods used to monitor minimal residual disease (MRD), as well as the status of the host immune system soon after the autograft and during the clinical follow-up all need to be studied.

In the present report, we show the findings of a pilot study to evaluate the results of a high-dose chemotherapy program followed by unmanipulated PBSC reinfusion in 20 CLL patients in CR after fludarabine treatment, as well as the follow-up monitoring of MRD and immunologic reconstitution.

Design and Methods

Patients' characteristics

All patients with advanced CLL (Binet stage B and C) less than 55 years old who achieved clinical CR after fludarabine were offered a program of autologous unmanipulated PBSC transplant after the BEAM (BCNU, etoposide, ara-C, melphalan) conditioning regimen.[20] Allogeneic bone marrow transplant was not considered even in patients with a HLA compatible sibling. Patients were required to have a good performance status with no severe concomitant medical or psychiatric illnesses. From April 1995 to August 1999, 20 unselected CLL patients were enrolled in the study. The clinical features of the patients and their disease status at the time of enrollment are reported in Tables 1 and 2. Their median age was 46.5 years (range 21 to 58); 17 were males and 3 females. The diagnosis was based on the criteria recommended by the National Cancer Institute Sponsored Working Group.[21] Patients were classified as having CLL on the basis of May-Grünwald Giemsa stained peripheral blood films and on positivity for CD20, CD5 and CD23 (Becton Dickinson, Mountain View, CA, USA). B-cell clonality was established by immunoperoxidase[22] using anti-κ and anti-λ light chain polyclonal antibodies (Ab) (Becton Dickinson). Due to the weak surface Ig expression, cytoplasmic evaluation either by flow cytometry or immunoperoxidase was carried out. Responses were scored according to the National Cancer Institute-Sponsored Working Group recommendations:[21] a patient was considered in CR in the absence of constitutional symptoms, lymphadenopathy and organomegalies, associated with a normalization of blood counts and a normal BM biopsy or a BM lymphocyte infiltration < 30%. Prior to fludarabine treatment, all patients were in advanced clinical stage (Binet stage B, 18 patients; Binet stage C, 2 patients) (23). At the time of transplant, 10 patients were in 1st clinical CR achieved after fludarabine plus prednisone ± interferon alpha and 10 were in ≥ 2nd CR after fludarabine ± ara-C, mitoxantrone and dexamethasone.[24]

The first evaluation was performed 2 months after stem cell infusion, while follow-up restagings were carried out every 4 months or, more frequently, when clinically indicated. Clinical relapse was based on the same criteria utilized for diagnosis.[21]

Evaluation of MRD and the pattern of immunologic reconstitution post-transplant were performed at different time intervals, as described below.

Informed consent was obtained from all patients.

Mobilization regimen and leukapheresis

The mobilization regimen consisted of cyclophosphamide (Cy) at 7 g/m^2 followed by glycosylated recombinant human granulocyte colony-stimulating factor (rhG-CSF, kindly provided by Italfarmaco, S.p.A., Milan, Italy) at a dose of 5 µg/kg/day s.c. starting on the first day after Cy until the end of the PBSC collection. The optimal timing of PBSC collection was determined on the basis of the evaluation of circulating CD34$^+$ cells; leukapheresis were started when the blood CD34$^+$ cells had risen to >10/µL and continued until the target of 2×10^6 CD34$^+$ cells/kg was reached. All procedures were performed using the Cobe Spectra cell separator (Cobe, Lakewood, CO, USA); 7 to 12 liters of blood were processed with a flow rate set at 50-90 mL/min and the collection pump set at 1.0 mL/min. All patients had a single or double-lumen subclavian vein catheter placed which was utilized only to return the processed blood; a large-bore peripheral venous needle was inserted in all patients. Collection products were cryopreserved and stored in liquid nitrogen until use.

Table 1. Patients' caracteristics.

Pt.	Age/sex	Stage at FLU treatment	Therapies prior to PBSC mobilization	Time (mos.) from FLU to PBSC mobilization	Time (mos) onset to autograft
1	39/M	II B	FAMP × 6 FAMP × 3	6	29
2	44/M	II B	VCR + CHL + PDN × 2 VCR + CHL + PDN × 2 VP-16 + CTX + PDN × 4 CHL + PDN × 6 HDCTX × 2 FAMP × 6 FANS × 4	6	67
3	51/M	II B	FAMP × 6	2	52
4	53/M	II B	FAMP × 6	3	11
5	43/F	I B	FAMP × 6	4	35
6	44/M	II B	FAMP + IFN × 6 IFN CHL + PDN × 10 Splenectomy FANS × 4	1	29
7	44/M	IV C	FAMP × 6 SPLENECTOMY FANS × 4	2	22
8	46/M	II B	CHOP × 6 IFN x 18 months FANS × 6	2	52
9	22/M	NE	CHOP + BLEO × 4 FANS × 2	2	11
10	53/M	IV C	FAMP × 6	4	11
11	36/F	I B	CHL + PDN FAMP × 6	2	132
12	61/M	II B	FAMP × 6 FANS × 6	3	95
13	53/F	II B	FAMP × 6	9	48
14	47/M	II B	FAMP × 5	7	57
15	57/M	II B	FAMP + IFN × 6 FANS × 4	2	24
16	43/M	II B	IFN + FAMP × 6	2	38
17	57/M	II B	FAMP × 6	2	40
18	60/M	I B	FAMP × 6	1	19
19	60/M	II B	RT (spleen) Splenectomy FAMP+IFN × 6 FANS × 3	3	118
20	60/M	II B	FAMP+IFN × 6	2	23

FAMP = Fludarabine + Prednisone; FANS = Fludarabine + Mitoxantrone + Prednisone; CHOP = Cyclophosphamide + Vincristine + Adriamycin + Prednisone; VCR = Vincristine; CHL = Chlorambucil; PDN = Prednisone. IFN = Interferon; CTX = Cyclophosphamide; VP-16 = Etoposide. BLEO = Bleomycin.

Conditioning regimen and supportive care

The preparative regimen was identical for all patients and consisted of the BEAM protocol.[20] Within 24 hours of the end of chemotherapy,

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Table 2. Disease status at enrollment.

Pt.	BM biopsy: pattern	BM biopsy: lymph. (%)	CD5/CD20+ cells/BM lymph. (%)	PB lymph. (x 10^9/L)	CD5/CD20+ cells/PB lymph. (%)
1	Int	10	90	740	35
2	Int/Nod	30	5	930	1.52
3	Int	15	2	360	6
4	Int	30	4.3	590	9.8
5	Int	10	10	600	9.8
6	Int	10	26	2,400	18.3
7	Nod	20	24.7	800	5
8	Nod/Int	10	7.2	1,000	1.6
9	Int	10	0	300	3
10	Int	20	27	1,200	7
11	Nod	19	1.5	400	0.6
12	Int	10	7	900	2
13	Nod	20	19	300	20
14	Int	10	24	500	16
15	Int	10	8	1,200	3
16	Int	15	19	800	9
17	Int	10	0	380	0
18	Int/Nod	10	5	300	2
19	Int	<10	2.6	2,000	8
20	Nod	30	4	1,000	0

BM: bone marrow, PB: peripheral blood; Lymph.: lymphocytes; Int: interstitial, Nod: nodular.

patients received cryopreserved autologous stem cells. After reinfusion, patients received rhG-CSF (5 µg/kg/day) until neutrophils exceeded 0.5×10^9/L on three successive days.

Prophylactic oral ciprofloxacin and intravenous acyclovir (15 mg/kg/day) were given routinely during aplasia, and broad spectrum i.v. antibiotic therapy was instituted in the presence of fever >38°C. All blood products administered were irradiated with 20 Gy before infusion. Platelet transfusions were administered when the platelet counts were $<10\times10^9$/L or when clinically indicated.

Immunophenotypic analysis

CD34+ cells were enumerated in unseparated PB or in leukapheresis bags using a PE-conjugated anti-CD34 monoclonal Ab (MoAb; HPCA-2, Becton Dickinson) and a FITC-conjugated anti-CD45 MoAb (Becton Dickinson), according to published methods.[22] Briefly, 3×10^5 cells were suspended in 80-100 µL of phosphate buffered saline (PBS) containing 0.1% sodium azide (PBS-NaN3). Cells were incubated for 30 min at 4°C, washed twice in PBS-NaN_3 and incubated in NH_4Cl for 15 min to lyse erythrocytes. Isotype and fluorochrome-matched irrelevant MoAb were used as controls. For the evaluation of MRD, mononuclear cells were incubated with FITC conjugated anti-CD20 and PE-conjugated anti-CD5 MoAb, as above. The κ/λ ratio was established as previously described. When a proportion of CD20/CD5+ cells >10% was found, the clonality was investigated on the basis of the Ig κ/λ ratio.

Flow cytometry was performed on a FACScan or FACSCalibur (Becton Dickinson) equipped with a 15 mW air cooled argon-ion laser tuned at 488 nm. A minimum of 30,000 cells was acquired for each measurement. Data were analyzed using the FACScan or Cell Quest Becton Dickinson softwares.

A patient was considered in immunologic relapse of disease in the presence of >10% CD20/CD5+ cells associated with a documented Ig κ/λ light chain restriction.

Rearrangement of the JH locus

High molecular weight DNA was obtained from mononuclear BM and/or PB cell pellets following proteinase K digestion and salting-out extraction.[25] Polymerase chain reaction (PCR) was performed essentially as described by Saiki *et al.*[26] Thirty-five cycles of PCR were performed amplifying 1 µg of genomic DNA with 25 pmol of the amplimers CA1 sense and CA2 antisense recognizing VH-DH and JH consensus sequences.[27,28] The sequences of the primers used were:

5'-CCGAGGACACGGCCGTGTATTACTG-3' CA1
(3' end of the FR3 of VH genes);
5'-AACTGCTGAGGAGACGGTGACC-3' CA2
(3' end of the JH segments).

The PCR conditions were as follows: 95°C x 30 sec; 58°C x 30 sec; 72°C x 30 sec. Polyclonal and monoclonal JH- samples were always analyzed in parallel with the DNA tests as controls of the experimental conditions. A negative control consisting of all PCR reagents without DNA template was always added in each test. PCR products were denaturated in a denaturing dye at 100°C for 5 minutes, electrophoresed through a 10% non-denaturing polyacrylamide gel at 150V and evaluated after.

JH PCR sensitivity. To establish the sensitivity of this PCR method, we serially diluted DNA from CLL with DNA from normal bone marrow mononuclear cells prior to PCR amplification. Against the background of polyclonal rearrangements we could readily distinguish the clonal product in a dilution of 10^{-3}.

Statistical methods

Descriptive statistics based on ranges (such as the median, minimum and maximum) were used to analyze time to engraftment and OS. Survival curves were plotted according to the Kaplan-Meier method.

Results

PBSC collection

All patients completed the planned mobilization scheme with high-dose Cy and rhG-CSF, and were submitted to the apheresis procedures. No major toxicities were observed. After mobilization treatment a median of 3.47 (range 2.06-11.3) CD34+ cells were collected with a median of 1 (range 1-7) leukapheresis procedure in 15 out of 20 patients. Aphereses were started a median of 12 (range 10-14) days after Cy administration. Five of the 20 patients failed to mobi-

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lize the target number of CD34+ cells and 4 of them underwent a marrow collection and reinfusion (total number of nucleated cells reinfused 1.6, 1.7, 1.9 and 3.26×10^8/kg). In 1 patient from whom a low number of circulating CD34+ cells was collected (1.5×10^6/kg), BM was harvested for rescue; it was, however, not utilized because normal post-transplant engraftment was achieved following reinfusion of PBSC alone. Overall, a total of 16/20 patients were reinfused with PBSC (Table 3). Mobilization of CD34+ cells did not appear to correlate with the type of treatment previously received or with the time from the last chemotherapy cycle.

Engraftment

All patients engrafted; the median time to a granulocyte count greater than 0.5×10^9/L and to a platelet count over 20×10^9/L was 12 (range 9-24) and 15 days (range 10-115), respectively (Table 3). In 4 and 5 patients erythrocyte and platelet transfusions, respectively, were not required; one patient required no transfusion support. No engraftment delay was observed in patients heavily treated prior to mobilization therapy.

Toxicity

Fifteen patients developed fever during aplasia with documented bacteremia in 11 cases, related to the i.v. catheter in 3. In all cases, fever responded promptly to antibiotic therapy. Patients were discharged from the hospital after a median of 29.5 days (range 23-41). Long-term complications included cutaneous Herpes zoster infections in 4 patients 2, 3, 4 and 5 months after transplant, and a Guillan-Barre' syndrome that developed in 1 patient 3 months after transplantation. This patient was treated with plasma-exchange procedures and obtained full recovery from the immunologic disorder.

One patient died 60 days after autograft in complete hematologic reconstitution because of severe intestinal cryptosporidiosis and systemic candidiasis. Sixteen months after transplantation, a diagnosis of acute myeloid leukemia (AML) was made in one patient (#4, Figure 1) with no hematologic and immunophenotypic evidence of CLL. On molecular grounds, this patient always proved positive. The patient obtained a CR from the AML after FLAG (fludarabine, ara-c, rhG-CSF) therapy,[29] but one month later died of acute respiratory distress syndrome and, at autopsy, a concomitant metastatic lung cancer was found.

Table 3. PBSC collection and hematologic reconstitution.

Pt.	Apheresis number	CD34+ cells collected (x 10^6/kg)	Stem cell source	Days for PMN > $0.5x10^9$/L	Days for PLTS > $20x10^9$/L	Days of hospitalization
1	7	< 2	BM	21	115	40
2	3	11.3	PBSC	12	13	24
3	5	1.5	PBSC	15	63	28
4	7	4.8	PBSC	9	55	33
5	2	< 2	BM	24	52	35
6	4	3.55	PBSC	12	14	30
7	1	< 2	BM	24	32	29
8	5	3.9	PBSC	15	31	30
9	1	2.97	PBSC	13	14	25
10	1	2.79	PBSC	12	13	26
11	3	2.43	PBSC	11	35	23
12	1	8.6	PBSC	11	13	29
13	2	< 2	BM	22	34	41
14	1	3.4	PBSC	11	14	23
15	1	4.5	PBSC	12	15	28
16	1	2.39	PBSC	14	15	30
17	1	4	PBSC	11	10	37
18	1	3.55	PBSC	12	15	30
19	3	2.06	PBSC	12	12	33
20	1	2.25	PBSC	10	10	23

Clinical and biological follow-up

Overall, 18 patients are alive a median of 25 months (range 11-52) after transplant and 73 months (range 29-133) after diagnosis. Of these, 13 are in continuous hematologic CR, whereas 5 patients (#1, 2, 5, 6 and 10, Figure 1) had hematologic relapse 26, 33, 39, 40 and 26 months after transplantation. The OS probability is 0.87±0.04 projected to 52 months from transplantion (Figure 2).

Longitudinal molecular and immunologic monitoring of MRD was performed in all patients but one (#11, Figure 1) who died early after transplant due to infection. After autografting, 15 out of 19 patients were PCR negative, whereas the 4 remaining cases (#1, 2, 4, 12, Figure 1) were PCR positive on repeated determinations. With regard to the group of 15 PCR negative patients, 12 (#3, 7, 8, 9, 13, 14, 15, 16, 17, 18, 19, 20, Figure 1) remain persistently PCR and immunologically negative after a median interval of 17 months from transplant (range 11-49), while 3 patients (#5, 6, 10, Figure 1) converted to a PCR positive status 28, 24 and 16 months after their autograft. In all three patients an immunologic relapse was documented (7, 10, and 6 months after conversion to PCR positivity), while clinical relapse occurred 4, 6 and 4 months after the immunologic relapse and 39, 40 and 26 months after transplantation respectively.

Of the 4 CLL patients who were persistently PCR positive after transplantation, hematologic relapse was documented in 2 (#1 and 2, Figure 1), 26 and 33 months after autografting. In both cases, an immunologic relapse was demonstrated 12 and 7 months prior to the overt disease recurrence. Of the remaining patients, one (#4, Figure 1) died of another tumor, developed 16 months after his autograft, while in hematologic and immunologic CR from CLL, and the other (#12, Figure 1) became immunophenotypically positive 11 months after the transplant, while still in hematologic CR. Samples were taken simultaneously from both BM and PB in 39 instances. PCR analyses showed discordant results in 2/39 pairs, in both cases being positive in the BM and negative in the PB (#5 and 10) (Figure 1).

Interestingly, in 4 cases (#3, 7, 8 and 13) with a proportion of CD20/CD5+ cells between 12 and 32% no evidence of Ig light chain restriction was found.

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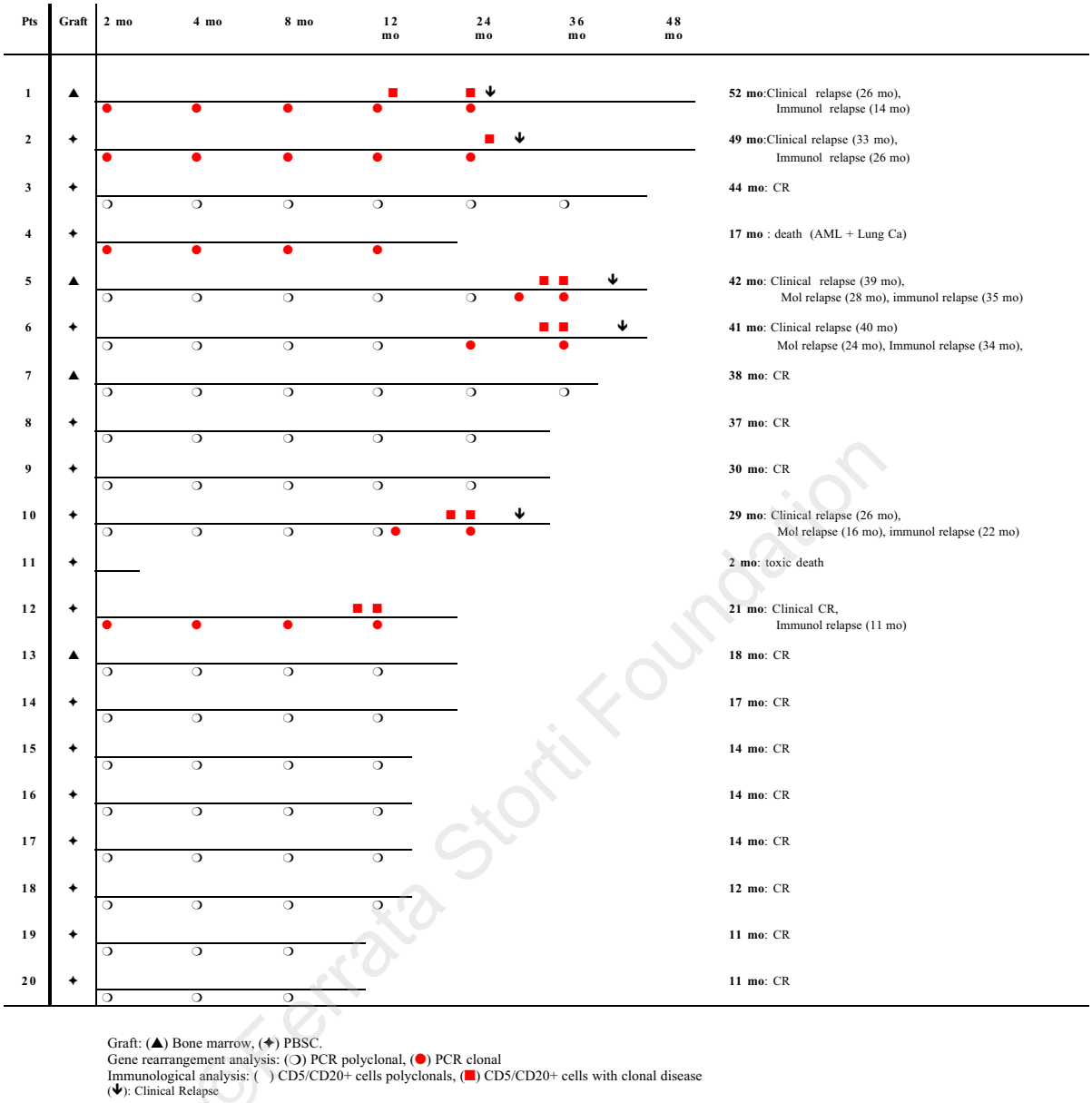

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Figure 1. MRD monitoring and follow-up.

This correlated with the molecular pattern. It is worth noting that patient #7 who on 7 consecutive determinations showed a proportion of $CD20^+/CD5^+$ cells between 15 and 32%, proved non-clonal, is at present in immunologic and molecular CR 38 months after autografting.

Eleven patients (#3, 7, 8, 9, 13, 14, 15, 16, 18, 19 and 20, Figure 1) at present still appear to be free of any clinical, molecular or immunologic evidence of disease either in the BM or in the PB. It should, however, be noted that only 4 patients have been followed up for longer than 24 months after receiving their autograft.

Immunologic reconstitution

The absolute number of $CD20^+/CD5^-$ lymphoid cells in the PB was markedly decreased after transplantation and gradually recovered at various time intervals ranging from 12 to 24 months. Twelve months after autograft, the median absolute number of circulating $CD20^+/CD5^-$ cells was 128 (range 2-756) for the 17 evaluable patients and, at 24 months, 180 (range 30-690) for the 9 evaluable patients (normal values: median 300/µL, range 170-620). In particular, a normal value of $CD20^+/CD5^-$ cells in the PB was restored in 47% and 90% of patients 12 and 24 months after their autograft, respectively. In the 5

Table 4. PB immunologic reconstitution - % ($\times 10^9$/L).

	2 months				12 months				24 months				36 months			
Pt.	CD3	CD3/CD4	CD3/CD8	CD4/CD8+ ratio	CD3	CD3/CD4	CD3/CD8	CD4/CD8+ ratio	CD3	CD3/CD4	CD3/CD8	CD4/CD8+ ratio	CD3	CD3/CD4	CD3/CD8	CD4/CD8+ ratio
1	ND	ND	ND	28 (224)	2 (16)	46 (368)	0.04		14 (378)*	2 (54)*	6 (162)*	0.33*	Cl. Rel.	Cl. Rel.	Cl. Rel.	Cl. Rel.
2	ND	ND	ND	92 (1840)	25 (500)	70 (1400)	0.36		71 (1597)	5 (112)	48 (1080)	0.10	Cl. Rel.	Cl. Rel.	Cl. Rel.	Cl. Rel.
3	86 (1229)	ND	ND	71 (1157)	19 (309)	53 (863)	0.35		60 (640)	4 (40)	33 (330)	0.12	70 (144)	34 (408)	55 (660)	0.6
4	40 (400)	ND	ND	55 (1210)	13 (286)	45 (990)	0.28									
5	45 (450)	2 (20)	38 (380)	0.05	60 (1020)	20 (340)	29 (493)	0.68	65 (1235)	24 (456)	47 (893)	0.51	47 (1222)*	21 (546)*	23 (598)*	0.9*
6	63 (2331)	2 (74)	49 (1813)	0.04	30 (780)	8 (208)	19 (494)	0.42	54 (2397)	18 (792)	47 (2068)	0.38	42 (1428)*	16 (544)*	24 (816)*	0.6*
7	69 (1676)	21 (510)	49 (1190)	0.42	57 (1140)	3 (60)	52 (1040)	0.06	60 (1140)	22 (528)	46 (1104)	0.47	47 (1786)	17 (646)	27 (1026)	0.6
8	99 (1386)	17 (238)	79 (1106)	0.21	42 (1260)	4 (120)	27 (810)	0.15	58 (1160)	17 (306)	32 (640)	0.47	69 (1173)	25 (425)	40 (680)	0.6
9	36 (360)	2 (20)	30 (300)	0.07	64 (768)	17 (204)	29 (349)	0.58	69 (1242)	29 (522)	35 (630)	0.82				
10	42 (714)	11 (187)	28 (476)	0.39	49 (945)	22 (462)	19 (399)	1.1	43 (1591)*	28 (1036)*	13 (481)	2.1				
11	ND	ND	ND													
12	79 (896)	27 (297)	45 (495)	0.6	65 (1755)*	24 (648)*	40 (1080)*	0.6*								
13	20 (40)	2 (4)	17 (34)	0.12	58 (696)	30% (360)	28% (336)	1.07								
14	69 (897)	12 (156)	56 (728)	0.21	71 (1775)	15 (375)	57 (1425)	0.26								
15	94 (1504)	19 (304)	76 (1216)	0.25	72 (792)	27 (297)	45 (495)	0.6								
16	89 (979)	8 (88)	78 (858)	0.10	72 (576)	22 (176)	48 (384)	0.45								
17	53 (106)	32 (64)	20 (40)	1.6	60 (240)	34 (136)	25 (100)	1.36								
18	93 (3069)	18 (594)	72 (2376)	0.25	86 (2408)	21 (588)	60 (1680)	0.35								
19	92 (828)	14 (126)	79 (711)	0.18												
20	86 (1634)	11 (209)	74 (1406)	0.15												
Median	69 (897)	12 (156)	49 (728)	0.21	60 (1020)	20 (297)	45 (495)	0.42	Median 60 (1235)	18 (456)	35 (640)	0.47	47 (1222)	21 (544)	27 (680)	0.6
N.V.	70 (1600)	44 (900)	29 (600)	1.2												

**Immunologic relapse.*

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patients evaluable 36 months after transplantation, only 1 (#3) had not recovered a normal value of $CD20^+/CD5^-$ cells.

In the post-transplant monitoring, the overall proportion and absolute number of circulating $CD3^+$ T-lymphocytes showed only a modest decrease compared to normal PB lymphocytes (Table 4). A persistently reversed CD4/CD8 ratio was found 2, 12 and 24 months after autografting, though a progressive trend towards an increased ratio was observed over time. The reduced CD4/CD8 ratio was due to a persistent decrease in the percentage and absolute number of $CD4^+$ T-lymphocytes. It should, however, be noted that a progressive and notable increase in the number of $CD4^+$ cells was recorded 12 and, to a further extent, 24 months after engraftment. The values, nonetheless, remained well below those of normal controls. A further increase in the absolute number of $CD4^+$ cells was also observed 36 months after transplantation in the 5 evaluable patients, in the presence of a persistently inverted CD4/CD8 ratio. Values at 36 months were not considered for the 2 patients (#1 and 2) who were in full hematologic relapse. The proportion of $CD8^+$ lymphoid cells always remained higher than that in normal controls at all post-transplant determinations. In most cases this translated into an increase in the absolute number of circulating CD8+ T-lymphocytes (Table 4).

The pattern of immunologic reconstitution was also analyzed in the BM in the post-transplant follow-up and a profile similar to that observed on circulating lymphocytes was found (data not shown).

Discussion

Autografting procedures are being increasingly performed in younger patients with high risk CLL with the aim of prolonging survival and, possibly, of eradicating the disease. However, the role of transplantation in the management of CLL has not yet been fully established and only a few reports dealing with this approach have been so far published.[5-19] In the present study, we analyzed a consecutive group of patients, homogeneous with respect to both selection criteria and transplant procedure. At the time of enrollment, all patients were in clinical CR following fludarabine therapy and were submitted to the same

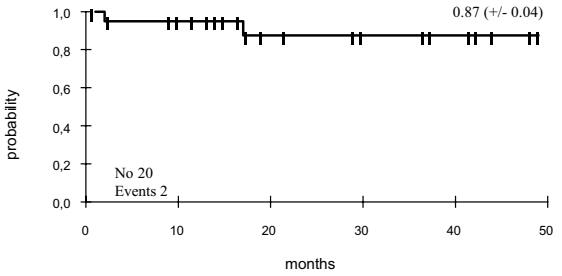

Figure 2. Overall survival probability.

PBSC collection and reinfusion program. Our data show that, in the majority of patients studied, therapy with nucleoside analogs did not prevent the successful harvesting of PBSC and indicate that the mobilization of $CD34^+$ cells and the time to post-transplant recovery do not correlate with previously received treatment.

Our experience confirms that the use of PBSC shortens in CLL, as in other conditions,[30-33] the duration of cytopenia reducing the associated complications, such as susceptibility to infections and transfusion requirement. A durable engraftment was always achieved and only one patient was lost due to post-transplant infection. At the latest follow-up, 18/20 patients are alive and well a median of 25 months (range 11-52) after transplantation; most patients have a good quality of life and have resumed their working activities. Thirteen patients are in continuous hematologic CR, while 5 have shown a clinical relapse at 26, 26, 33, 39 and 40 months from transplant.

In order to define the behavior of the leukemic clone over time more precisely, MRD after transplant was assessed in all patients every 4 months by immunophenotypic and molecular methods in both the PB and BM. Despite the use of unmanipulated PBSC, autografting allowed molecular and clinical remissions in high risk CLL patients responsive to fludarabine therapy. Molecular remissions were observed in 78% of patients, with a median duration of molecular remission of 17 months (range 11-49) after transplantation. In this respect, we underline that the sensitivity of our method is 10^{-3}, which is probably less than our method employing patient specific oligoprimers. However, using our PCR methodology we were able to predict immunologic and clinical relapse. In fact, the persistence or reappearance of JH clonality after transplant correlated with subsequent clinical relapse of the disease. This latter was also always preceded by an immunologically defined detection of CLL cells. It should, however, be noted that in patients with a higher likelihood of relapse, due to the persistence of MRD, autografting may still allow a durable clinical remission, with an interval from transplant to relapse of up to 33 months. Furthermore, two relapsed patients (#1 and 2, Figure 1), who were refractory to treatment prior to fludarabine, achieved a clinical remission that lasted more than 2 years and, at the time of post-transplant relapse, responded to conventional treatment.

Our study also shows that positivity of a previously negative tumor-specific PCR signal during the clinical follow-up anticipates immunologic recurrence of disease with an interval from molecular to immunologic relapse which ranges between 6 and 10 months. In conclusion, these data confirm the clinical relevance of PCR monitoring in CLL.[10]

It should be noted that in our experience MRD monitoring based on CD20/CD5 positivity may be associated with an apparent recurrence of disease which was ruled out by the lack of κ:λ Ig light chain restriction and confirmed by a non-clonal molecular pattern.

Taken together, these data indicate that monitoring of MRD in CLL patients autografted with PBSC is important to verify over time the disappearance or not of the leukemic clone and to identify early recurrence of disease. In our experience, persistence or reappearance of the molecular signal after engraftment predicts a subsequent immunologic and clinical CLL recurrence.

In view of the well documented abnormalities which affect the immunologic compartment of CLL patients[34] and of the known marked and prolonged down-modulating effects exerted by fludarabine on the CD4 lymphocyte subset of treated patients,[35, 36] the immune reconstitution during the clinical follow-up of patients autografted after fludarabine therapy was carefully monitored. Our data show an early decrease in $CD20^+/CD5^-$ non-leukemic B-lymphocytes that appears to normalize in most cases between 12 and 24 months after autograft. With regard to the T-cell compartment, despite an overall near-normal number of $CD3^+$ T-lymphocytes, a marked decrease in $CD4^+$ cells and concomitantly reversed CD4/CD8 ratio was recorded 2, 12, 24 and 36 months after engraftment. Despite this marked and persistent impairment in the longitudinal follow-up, a trend towards an increase in the number of circulating CD4 lymphocytes and, thus, of the CD4/CD8 ratio was observed over time. Despite these notable perturbations of the host immune system, no severe viral and/or fungal infections were observed after transplant; this prolonged immune impairment after autografting may, however, translate into diminished control of the growth of malignant cells of both the primary tumor, and other malignancies. It should be recalled that 1 of our patients developed two other neoplasms during the post-transplant follow-up.[37]

In conclusion, despite the use of unmanipulated PBSC the finding of a durable DFS is encouraging and suggests that autografting may represent a therapeutic option for this group of patients. However, the exact role of PBSC in the management of CLL will be conclusively established only through controlled studies aimed at answering different issues - e.g. clinical benefit compared with conventional chemotherapy, timing of transplantation, best conditioning regimen, type of purging, clinical meaning of MRD, role of immunologic reconstitution, management of relapse - that this procedure poses in CLL.

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GM, FRM and RF supervised the study over a 4-year period; AP, MV and SC were responsible for data collection and analysis; PA was responsible for cryopreservation; GC and GR were responsible for the molecular biology; IC and PdF were responsible for the immunology. FM chaired the study. The manuscript was prepared by GM and AP and reviewed by all the other authors.

Disclosures

Conflict of interest: none

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Potential implications for clinical practice

- High-dose chemotherapy followed by autologous PBSC reinfusion can be delivered to CLL patients less than 60 years of age with low transplant-related toxicity.
- The monitoring of MRD in CLL patients autografted with PBSC is important to verify over time the disappearance or not of the leukemic clone and to identify early recurrence of disease.
- The long-lasting impairment of the host immune repertoire after fludarabine followed by autograft has to be taken into account in the patients' management.

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