

Characterization of peripheral blood steady-state progenitor cells preserved in liquid culture conditions with or without GM-CSF and IL2

D. Sawadogo (1) (3), M^a J. Martínez (1), J. Merino (2), M^a L. Subirá (2), A. Brugarolas (1)

Departments of Oncology (1) and Immunology. (2) Clínica Universitaria. Facultad de Medicina. Universidad de Navarra. Duni Sawadogo. Service d'Hematologie. Centre Hospitalier Universitaire de Yopougon. 08BP2308. Abidjan 08. Côte d'Ivoire. (3)

SUMMARY: The cellular characteristics of steady-state peripheral blood progenitor cell (PBPC) apheresis, including total number of lymphomononuclear cells, CD34 and CFUs, was evaluated in a group of 26 chemo-radiotherapy patients as well as in a group of 23 surgically resected cancer patients. Three-to seven-day incubation in standard liquid culture conditions with growth factors (IL2, GM-CSF or both) correlated with a statistically significant increase in CD34+ and CD56+ cell populations compared with incubation without growth factors, especially when both GM-CSF and IL2 were used. In addition, an increase in CD33+, CD13+ and HLA-DR+ cell populations was observed after 3-7 days incubation with GM-CSF. The basal culture control exhibited a decrease in CD33+ and CD13+ cell populations while CD34+ and CD56+ cell populations were maintained. These results were similar in the treated and untreated groups of patients.

The infusion of GM-CSF and IL2 preincubated PBPC after intensive chemotherapy was associated with a rapid hematological recovery with a median time duration for WBC <500/uL, WBC <1.000/uL and platelets <20.000/uL of 7.9 days, 14.9 days and 10.7 days respectively.

We conclude that a short GM-CSF and IL2 preincubation of steady-state PBPC is associated with an increase in cell populations exhibiting the immune and progenitor cell phenotypes and correlates with an early hematological recovery after intensive chemotherapy.

(Rev Med Univ Navarra 1996; 40: 7-14).

Correspondencia: A. Brugarolas

Dept. Oncología
Clínica Universitaria
Universidad de Navarra
31080 Pamplona, Spain

Introducción

Peripheral blood became an alternative source of hematopoietic progenitors for autologous bone marrow transplantation (ABMT) because it was demonstrated that circulating stem cells reconstitute myelopoiesis after intensive chemotherapy (1-7).

The initial interest in peripheral blood stem cell (PBPC) autotransplant increased with the development of high-yield cell collection techniques at the time of hematological recovery after cytotoxic chemotherapy or following the administration of hematopoietic growth factors (8-11), and it is now well known that the use of PBPC harvested after mobilization with chemotherapy or cytokines markedly decreases the time to engraftment as compared to ABMT (12, 13).

However, Lobo et al showed that the addition of steady-state PBPC to ABMT products did not reduce the duration of pancytopenia (14). Their findings contrasted with those of other contemporary investigations which reported an improved recovery time by using mobilized PBPC (15).

Although these results have hampered the clinical use of non-mobilized PBPC, recent technological advances in stem cell selection, ex-vivo modulation and expansion, as well as the growing applications of allogenic PBPC transplant may expedite a renewed interest in their development.

The objective of this study was to investigate the phenotype of the steady-state PBPC in cancer patients and also evaluate the changes occurring when the PBPC are maintained for 3-7 days in liquid culture conditions with and without the addition of growth factors (GM-CSF and IL2). A preliminary study from our group had suggested an expansion effect upon CD34 and CD56 cell populations (16) and this research was designed as an in-depth confirmatory trial.

Patients and methods

Two series of patients were investigated in this study. The first series consisted of 26 patients with advanced or metastatic biopsy proven solid tumors, undergoing apheresis prior to intensive chemotherapy and PBPC support. Nineteen of these patients were heavily pretreated with chemotherapy and radiation therapy and the remaining seven patients, diagnosed as having malignant glioma, entered the study after debulking surgery. In this series of patients, steady-state aphereses were performed at least 4 weeks after the last dose of conventional chemotherapy. Samples for this study were drawn from all the apheresis products.

The second series was obtained from a group of 23 patients with locally advanced pathologically confirmed malignant tumors, who underwent lymphomononuclear cell (LMNC) apheresis in a surgical adjuvant program of adoptive immunotherapy. At the time of the study this group of patients had received minimal prior therapy and no previous cytotoxic drugs. In both series of patients aphereses were performed in steady-state conditions, without previous mobilization with chemotherapy or cytokines. Because of the different nature of the treatment in this second series, the samples were drawn from a single day apheresis.

The characteristics of the patients of both series are shown in Table 1. Selection criteria included: Karnofsky performance status (KPS) score >70%; adequate hematological function with WBC >3000/uL and platelets >75000/uL; normal hepatic function tests with bilirubin <1.5 mg/dL and liver enzyme profile <2 x normal values; normal renal function with creatinine <1.6 mg/dL and creatinine clearance >80 mL/min; normal cardiopulmonary status without previous history of myocardial ischemia, bronchial asthma or pulmonary obstructive disease; and good neurological and mental functions. All patients gave signed informed consent.

Intensive chemotherapy protocols for the first series of 26 patients included the following agents and doses: Cyclophosphamide, 160 mg/kg body weight, plus mitoxantrone, 40 mg/m², or thiotepa, 700 mg/m², for breast cancer patients; carmustine, 900 mg/m², plus intracarotid cisplatin, 60 mg/m², with simultaneous radiotherapy, up to 50 Gy in divided fractions of 200 cGy, five fractions a week, for five weeks, to the whole brain, in patients with malignant glioma; and carboplatin, 1500 mg/m², plus etoposide, 1200 mg/m², for patients with non-Hodgkin's lymphoma, multiple myeloma and ovarian cancer. All drugs were given in a 3

Tabla I

	SERIES	
	Intensive chemotherapy	Adoptive immunotherapy
Patients: Number	26	23
KPS (range)	80-100	80-100
Sex: M/F	10/16	14/9
Age: median (range)	46 (26-63)	53 (25-73)
Previous therapy:		
Surgery	20	23
Radiotherapy	6	0
Chemotherapy	19	0
Tumor Type:		
Ovarian Ca.	1	1
Multiple myeloma	1	-
Pancreas Ca.	-	1
NHL	5	-
Breast cancer	11	-
High grade glioma	8	-
Low grade glioma	-	3
Melanoma	-	8
Renal carcinoma	-	10

KPS: Karnofsky Performance Status.

hour intravenous infusion in two equally divided daily doses, beginning at the end of the last apheresis. Conventional antiemetic drugs were administered concurrently.

After PBPC infusion patients were admitted to standard hospital rooms. No special isolation procedures were carried out. Gut decontamination was accomplished with oral norfloxacin and nystatin. Rifampin was added to protect for staphylococcal species. Total parenteral nutrition was initiated if patients had had <500 kcal daily intake for more than 5 days. Parenteral broad spectrum antibiotics were begun for temperature >38.5°C and adjustments were carried out according to the clinical response and culture results. Toxicity was graded by the NCI-BMT toxicity criteria. WBC and platelets were evaluated daily using standard automated cell counters and PMN were evaluated when the WBC was >1000/uL. Serum creatinine and hepatic enzymes were evaluated every 2-4 days. Vancomycin and aminoglycoside antibiotics were prescribed according to their serum pharmacokinetic elimination parameters. Packed red cell and single or multiple donor platelet concentrates were administered to maintain hemoglobin >9.5 g/L and platelets >15000/uL, except in glioma patients where the mini-

imum desirable platelet count was $>25000/\mu\text{L}$. The company of a healthy relative or friend was allowed throughout the hospital stay.

Lymphomononuclear cell (LMNC) apheresis:

Patients underwent daily apheresis, repeated from Tuesday to Friday, until a cumulative minimum of LMNC $6 \times 10^8/\text{kg}$ body weight was harvested. If the required amount of LMNC was not collected in the four scheduled days, additional days of aphereses were planned, usually on Saturday and Monday, until the set amount was reached. If the required amount of LMNC was collected in less than four days, the program was not reduced and the planned four-day apheresis program was completed.

Aphereses were performed using the CS-3000 (Fenwall Laboratories, Deerfield, Ill) continuous cell separator. A modified L-1 mononuclear-cell collection program with red-cell interface set at 020 units was used. Daily aphereses lasted from 3 to 5 hours, during which time up to 9.9 L of whole blood was processed at a flow rate of 25-75 mL/min. Phereses were performed using percutaneously inserted double lumen central venous catheters. Harvested cells were collected in acid citrate dextrose (ACD, NIH Formula A) with a volume of 150-200 mL in a Fenwall transfer bag.

Liquid culture: Mononuclear cells were automatically distributed to B-L polyoleofin culture bags (PL-732; Fenwall Laboratories) at 3×10^6 cells/mL and incubated flat on perforated shelves without agitation at 37°C for 3 to 7 days in 5% CO_2 atmosphere. Culture

medium was RPMI 1640 (Gibco, Gaithersburg, MD, USA) which contained 2 mM glutamine, 50 $\mu\text{g}/\text{mL}$ gentamycin and fungizone 2.5 $\mu\text{g}/\text{mL}$ and 5% heat inactivated fetal calf (FCS) or human AB serum. When indicated the incubation medium contained 500 U/mL of rhIL-2 (specific activity 1.5×10^6 U/mL) (British Biotechnology, Oxford, UK), and 400 U/mL of rhGM-CSF (specific activity 100×10^6 U/mg) (Schering-Plough, Kenilworth, NJ, USA), or both rhIL2 and rhGM-CSF at the same previously mentioned doses.

A 2 mL aliquot was taken from the culture bags 24 hours before cell harvest and it was cultured and Gram-stained for the presence of microorganisms. Mononuclear cells were concentrated automatically in the CS-3000 cell separator and run normally with the centrifuge set at 1600 rpm. Cells were pumped at a flow rate of 88 mL/min from the bag into the component-rich line over the plasma pump and into the collection chamber. After the cells were concentrated they were repeatedly washed with saline (2 L) and then removed from the centrifuge. Human serum albumin, 12.5 g in 50 mL, was added to the 200 mL bag that contained the cells prior to infusion. Direct Gram stain of the concentrated product was carried out before the infusion. Mononuclear cells were administered intravenously through a large-bore central venous catheter over a 60-120 min period 48-72 hours after intensive chemotherapy.

Membrane antigenic markers: The monoclonal antibodies (MABS) employed in this study were Anti-

Tabla II

	BASAL PRIOR TO INCUBATION	MEDIUM ALONE	INCUBATION MEDIUM + GM-CSF ^a	MEDIUM +GM-CSF+IL-2 ^b
CD3	63.9 ± 0.9	65.6 ± 1.3	60.5 ± 1.7	56.9 ± 2.8
CD56	5.8 ± 0.5	5.3 ± 0.5	8.4 ± 0.7**	10.5 ± 0.9*
CD34	4.9 ± 0.5	4.7 ± 0.8	8 ± 0.6***	19.6 ± 2.8*
CD33	14.2 ± 0.7	9.5 ± 0.9***	17.5 ± 1.1*	16.2 ± 2.2
CD13	12.3 ± 0.9	8.2 ± 0.9**	16.4 ± 1***	15.7 ± 2+
CD15	5 ± 0.5	3.8 ± 0.4	5.1 ± 0.7	17.3 ± 2.9+
HLA-DR	10 ± 0.8	12.5 ± 1*	19 ± 1**	20 ± 1.8*

^a rhGM-CSF 400 U/106 cells/mL; ^b rhIL2 500 U/106 cells/mL.

* $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$. All p values are related to basal values.

+ The statistical power to analyse the differences was lost because some samples of this group were inadequate.

LEU-4 (CD3), LEU-19 (CD56), HPCA-2 (CD34) and HLA-DR from Becton Dickinson (Becton Dickinson Immunocytometry Systems, Erembodegen, Belgium), Anti MY7 (CD13) and MY9 (CD33) from Coulter (Coulter Immunology, Hialeah, FL) and anti-CD15 from Serotec (Serotec Ltd, Oxford, UK).

Appropriate isotype controls accompanied each analysis. All MABS were used as direct fluorescein isothiocyanate (FITC) conjugates at the concentrations recommended by the suppliers. Aliquots of 50 ml containing 5×10^5 cells were incubated with the MAB at 4°C for 30 min. After incubation, cell suspensions were washed twice in cold PBS-5% FCS. Cells were fixed in 0.5 ml of 2% phosphate-buffered formalin and submitted to flow cytometric analysis. A Coulter Profile Flow Cytometer (Coulter Electronics, Hialeah, FL) was used.

Statistical analysis: In both groups data concerning each patient were computed using the Wilcoxon Rank Sum test for median differences. All p values are two-sided. The relation between recovery time and progenitor cell assays, in the group of patients of the first series, was studied by use of regression analysis and correlation coefficient.

Results

The observed differences found in the two series of patients are shown in Table I and occurred mainly in the tumor type for which the indication of PBPC aphereses had been made. In the first series there is a predominance of breast cancer (11 patients), malignant glioma (8 patients), and lymphoma (5 patients), while

in the second series the most frequent diagnoses were renal cell carcinoma (10 patients) and melanoma (8 patients) and low grade glioma (3 patients). This accounted for the observed differences in age and sex distribution.

Each apheresis collected $0.9\text{--}2.1 \times 10^{10}$ LMNC, and the daily repetition of the procedures did not modify the daily mononuclear cell count although a transient decrease in platelet count was observed.

Phenotype: The median cell values for the samples obtained in the first series of patients were CD34+ 2.5% (range 1%-15%) and CD56+ 4.75% (range 1%-12%) in basal conditions. In the second series median values were CD34+ cells 2% (range 0%-19%), and CD56+ cells, 4% (range 1%-12%).

The results obtained after incubation and the statistical differences obtained in comparison with the basal samples are shown in Tables II and III. A mean of 4.7% of CD34+ cells were found in the series of 26 patients undergoing apheresis prior to high-dose chemotherapy and a mean of CD34+ cells 5.6% were found in the series of 23 patients in whom apheresis was performed for adoptive immunotherapy studies. This difference was not significant. In the first series of patients CD34+ cells had a statistically significant increase with the incubation with GM-CSF to 8% and with GM-CSF plus IL2 to 19.6%. In the other series of 23 patients in whom apheresis was performed for adoptive immunotherapy, CD34+ cells increased up to 13.6% with GM-CSF incubation.

The percentage of cells exhibiting the myeloid differentiation markers CD15 and CD33 significantly in-

Tabla III

	BASAL PRIOR TO INCUBATION	MEDIUM ALONE	INCUBATION MEDIUM + GMa	MEDIUM +IL-2b
CD3	68.8 ± 1.7	66.9 ± 2.3	64.5 ± 2.8	68.8 ± 2.3
CD56	4.6 ± 0.7	6.4 ± 0.9	5.7 ± 0.7	13.3 ± 1.1**
CD34	3.5 ± 1	5.6 ± 1.8*	13.6 ± 1.4**	5.2 ± 0.8*
CD33	8.5 ± 0.9	7.3 ± 0.9	11.8 ± 1.6	9.3 ± 1.9
CD13	7.7 ± 0.9	7.3 ± 0.8	12 ± 1.5	9.2 ± 1.5
CD15	3.3 ± 0.6	6.1 ± 1.7	7.3 ± 1.2**	4.3 ± 0.9
HLA-DR	10.6 ± 18	11.5 ± 0.8	14.1 ± 1.1*	12.6 ± 1.4

a rhGM-CSF 400 U/106 cells/mL; b rhIL2 500 U/106 cells/mL.

* p < 0.05; ** p < 0.005. All p values are related to basal values.

Tabla IV

			Median (range)
Collected CMN	x 10 ⁶ /kg body weight	10.8	(6.3 - 16.4)
" CFU-GM	x 10 ⁴ /kg body weight	1.9	(0-28.9)
" BFU-E	x 10 ⁴ /kg body weight	0.1	(0-28.7)
" CD 34+	x 10 ⁶ /kg body weight	6.07	(2.11-68.56)
Infused CD 34+	x 10 ⁶ /kg body weight	20.47	(10.59-119.25)
Time to hematological recovery:			
Days < 500 WBC/uL		7.9	
Days > 1000 WBC/uL		14.9	
Days > 20000 platelet/uL		10.7	

creased with GM-CSF incubation in the samples of the two series. These antigens were not significantly increased in the GM-CSF plus IL2 incubation in the first series or with single IL2 incubation in the second series.

Incubation with growth factors always led to an increase in immune cells wearing the CD56+ antigenic marker. Median basal value for CD56+ cells was 5.8%, and reached 8% with GM-CSF incubation and 10.5% with GM-CSF plus IL2 incubation in the first series of 26 patients with high-dose chemotherapy. In the second series of 23 patients with adoptive immunotherapy, incubation with IL2 induced a great increase in CD56+ cells, from a median basal value of 4.6% to 13.3%.

Clinical correlations (Table IV): The effect of PBPC incubation upon hematological recovery was studied only in the first series of 26 patients undergoing intensive chemotherapy and autologous peripheral blood progenitor cell transplantation. The median number of LMNC collected was 10.8 x 10⁶/kg body weight (range 6.3-16.4). This was usually accomplished in 4 aphereses each processing 10 L of blood. Only 2 patients required 5 aphereses to reach the set value in excess of 6 x 10⁶/kg body weight. Fifteen patients reached the minimum LMNC 6 x 10⁶/kg in three aphereses but, according to the protocol program, the fourth procedure was completed.

Median CFU-GM and BFU-E collected were 1.9 x 10⁴/kg (range 0-28.9) and 0.1 x 10⁴/kg (range 0-28.7) respectively.

CD34+ cells were calculated at the time of harvest and at the time of infusion after incubation with GM-CSF or GM-CSF plus IL2. The median values increased from 6.07 x 10⁶/kg (range 2.11-68.56) initially, to 20.47 x 10⁶/kg (range 10.59-119.25) at infusion. No

significant toxicity was associated to the cell infusion except of mild fever and chills.

There was no mortality during the period of aplasia in these series and only four patients had documented infection which was resolved with appropriate antibiotic therapy. No grade 3-4 extramedullary toxicity was observed. The median duration of neutropenia was 7.9 days for WBC <500/uL, 9.1 days for ANC <500/uL, 14.9 days for WBC <1000/uL, and 10.7 days for platelets <25000/uL.

No statistically significant correlations were identified between the time to hematological recovery and the number of cells infused. The observed lack of correspondence between the number of cells infused and the time to recovery applied to total LMNC cells, CFU-GM, BFU-E, CD34+, CD33+ or CD56 cells harvested and CD34+, CD33+ or CD56+ cells infused.

Discussion

We studied two series of patients, one heavily treated and another chemo and radiotherapy naive, and no significant differences were observed between the LMNC apheresis counts. Each apheresis collected 0.9-2.1 x 10¹⁰ LMNC, representing one-third to one-half of the LMNC cells present in the volume of blood processed. Daily harvesting did not modify the WBC count at the end of the procedure, possibly indicating a spontaneous physiological mechanism of cell mobilization although we could not find data on progenitor mobilization by the apheresis procedure itself in the literature. Clonogenic cells were scarce in steady-state peripheral blood and CD34+ cells represented a 0.6% of the LMNC, this figure being the same both in previously treated or untreated patients. Cell viability was excellent and the cell infusion was well tolerated with minor side effects characterized by transient fever and chills. There were no side effects attributed to FCS or cell product debris, probably because the repeated extensive cell washing prior to cell concentration and infusion.

The threshold values given for a successful hematologic reconstitution, according to estimates from the literature, indicate that a PBSC transplant requires, CFU-GM >20 x 10⁴/kg, and CD34+ cells >2.5 x 10⁶/kg body weight, these usually being obtained in >4 x 10⁸/Kg LMNC apheresis (17-21). In comparison with these recommended values the results obtained in our series exceeded the LMNC count and the CD34+ cell number (LMNC, 10.8 x 10⁶/kg, and CD34, 6.07 x 10⁶/kg) which were usually harvested in four conse-

cutive days without any special preparation. The median number of steady-state circulating CD34+ cells in our series was 0.6% (range 0.1-1.3%). The fact that pre-treated patients presented a little higher, although non-significant, CD34+ cell yield might be attributed to some delayed mobilization effect of conventional chemotherapy which was given four weeks prior to apheresis. The values of CFU-GM and BFU-E counts in our series were low (Table IV). These results are consistent with the literature: mobilized PBSC are rich in clonogenic cells but steady phase PBSC give rise to few CFU-GM and BFU-E, as occurred in our series (8, 9, 15, 22).

It has been suggested that CFU-GM counts cannot be used to compare engraftment in different series because results in different institutions are very variable. For this reason some authors suggested that the number of CD34+ cells is the best predictor of engraftment capacity (18, 22, 23). However other groups do not completely support this because the quantitative assessment of the leucapheresis product is difficult and both GM-CFC and CD34 cell assays are highly variable between laboratories (20, 24). For the same reason the LMNC count, having little specificity but being highly reproducible and simple to perform, is frequently used as a practical guideline to assess the quality of PBPC: A minimum amount of LMNC, 6×10^8 /kg, is generally adequate for non-mobilized PBPC, and less LMNC, 4×10^8 /kg, is acceptable for mobilized PBPC (23, 25, 26).

Pettengell et al were recently able to obtain successful engraftments with the product of a single apheresis obtained after an intensive priming with chemotherapy followed by mobilization with G-CSF (24). The apheresis product was considered adequate when concurrent counts demonstrated LMNC $>1 \times 10^8$ /kg, CFU-GM $>1 \times 10^6$ /kg, and CD34+ $>4 \times 10^6$ /kg.

We did not find any positive correlation between the collected or the infused number of LMNC or CD34 cells and the time to hematopoietic recovery. Mortimer et al have reported that when a very large number of CD34 cells are infused this test loses the predictive capacity and other factors influence the recovery (27).

In this study it was shown that GM-CSF and IL2 incubation increased CD34 and CD56 cell populations. Incubation with GM-CSF demonstrated an increase in CD34 cells and a rise in differentiated populations exhibiting CD33 and CD15 antigens. Haylock et al have similarly reported that ex-vivo incubation of peripheral blood CD34 cells with different combinations of cytokines, including IL1, IL3, GM-CSF, IL6, G-CSF and SCF, and more specially with the combined addition of the

six growth factors, enhanced the proliferation and differentiation effect as compared to the control group (28). According to this report CD34 cells disappeared after day 7-10, CD33 appeared from day 3-7 and the same occurred with the CD11b and CD15 cell populations, indicating an overall maturation effect (28).

The role played by GM-CSF in the survival and further commitment towards proliferation and differentiation of very early progenitor cells is not well understood. In-vivo cell kinetic studies have demonstrated that GM-CSF main activity is related to the recruitment of temporarily resting hemopoietic precursors and the acceleration of the cell cycle duration, which results in a very rapid increase of mature circulating cells (29). After a three day GM-CSF continuous infusion the in-vivo rate of cycling cells/hour increased from 1.3% to 3.4%, the cell cycle decreased from 86 hours to 26 hours, the number of circulating granulo-monocytes increased by a factor of 3 to 4, and the bone marrow demonstrated an increase in immature cycling cells and CFU-GM colonies by a factor of 2. These results suggest a relatively late role of GM-CSF in the hierarchical organization of hemopoiesis. A possibility of ligand-usurpation due to cross reaction between different growth factors and their receptors has been ruled out by Testa et al (30). It has been shown by these authors that CD34 cells exhibit different high affinity receptors, IL3 (2700/cell), GM-CSF (300/cell), IL6 (145/cell), and EPO (75/cell) all of which are downmodulated by their specific growth factor, and although cross-reactivity among receptors of GM-CSF, IL3, and to a lesser extent Kit ligand, do exist, the transactivation effect only can occur for the distal growth factors, that is, downstream towards more mature lineages (30).

Another possible mechanism of action of GM-CSF incubation is a protective biological effect by inducing the appearance of homing receptors, resulting in a greater seeding and grafting efficiency, as described by Tavassoli et al. (31). This group demonstrated that both IL3 and GM-CSF upmodulated the presence of a membrane homing protein which interacts with lineage specific stromal cells and supports the proliferation and maturation of hemopoietic stem cells.(32). In addition IL3, GM-CSF and G-CSF may play a trophic function, preventing cell death by suppression of apoptosis (33).

Some of these findings have been confirmed in animal models. A brief preincubation with growth factors led to the expansion of progenitor cells and reduced

the post-transplantation recovery period in mice (34). In addition, a recent clinical study indicated that the preincubation of marrow allografts with IL3 or GM-CSF enhanced the hemopoietic recovery, reducing the time of severe cytopenia by approximately one week (35). In this study, as well as in others (28, 36), PBPC products incubated with GM-CSF induced differentiation towards the myeloid series, as demonstrated by a rising count of cells carrying the CD33, CD15, and HLA-DR receptors. These investigations are in agreement with the in-vivo cytokine measurements after bone marrow transplantation. It has been demonstrated that one of the first growth factors produced is GM-CSF, which is released two weeks before the recovery of granulocytes, and that a subsequent G-CSF peak preceded polymorphonuclear cell recovery by one week (37). These findings, as well as our results, suggest that ex-vivo incubation of hemopoietic precursors with a combination of cytokines can induce

cell expansion and cell differentiation that could be clinically relevant.

In conclusion, the liquid culture and growth factor incubation of steady-state PBPC, apheresed in 4 consecutive days, seems to be a simple and dependable procedure for intensive chemotherapy transplantation studies. It may avoid a time-delay imposed by mobilization techniques and the need for serial cryopreservation procedures. In absence of a comparison study with other commonly used techniques we can only conclude that it appears effective and it could be used in patients lacking an adequate PBPC sample from previous mobilization procedures. Further studies are recommended.

Acknowledgements:

We acknowledge the financial support of the Fundación Echébano and the Centro de Investigaciones Biomédicas of the University of Navarra.

REFERENCES

- GOODMAN JW, HODGSON GS. Evidence for stem cells in the peripheral blood of mice. *Blood* 1962; 10:702-714.
- McKREDIE KB, HERSH EM, FREIREICH EJ. Cells capable of colony formation in the peripheral blood of man. *Science* 1971; 171:293-294.
- BARR RD, WHANG-PENN J, PERRY S. Hemopoietic stem cells in human peripheral blood. *Science* 1975; 190:284-285.
- BARR RD, McBRIDE JA. Hemopoietic engraftment with peripheral blood cells in the treatment of malignant disease. *Br J Haematol* 1982; 51:181-187.
- MCCARTHY MD, GOLDMAN JM. Transfusion of circulating stem cells. *Crit Rev Clin Lab Sci* 1984; 20:1-24.
- KÖRBLING M, DORKEN B, HO AD, PEZZUTTO A, HUNSTEIN W, FLIEDNER TM. Autologous transplantation of blood-derived hemopoietic stem cells after myeloablative therapy in a patient with Burkitt's lymphoma. *Blood* 1986; 67:529-532.
- KESSINGER A, ARMITAGE JO, LANDMARK JD, SMITH DM, WEISENBURGER DD. Autologous peripheral hematopoietic stem cell transplantation restores hematopoietic function following marrow ablative therapy. *Blood* 1988; 71:723-727.
- RICHMAN CM, WEINER RS, YANKEE RA. Increase in circulating stem cells following chemotherapy in man. *Blood* 1976; 47:1031-1039.
- ABRAMS RA, JOHNSTON-EARLY A, KRAMER C, MINNA JD, COHEN MH, DEISSEROTH AB. Amplification of circulating granulocyte-monocyte stem cells (CFU-C) numbers following chemotherapy in patients with extensive small cell carcinoma of the lung. *Cancer Res* 1981; 41:35-41.
- MOLINEUX G, POJDA Z, HAMPSON IN, et al. Transplantation potential of peripheral blood stem cells induced by granulocyte colony-stimulating factor. *Blood* 1990; 76:2153-2158.
- SOCINSKY MA, CANNISTRA SA, ELIAS A, ANTMAN KH, SCHNIFFER L, GRIFFIN JD. Granulocyte-macrophage colony-stimulating factor expands the circulating haemopoietic progenitor cell compartment in man *Lancet* 1988; 1:1194-1198.
- TO LB, HAYLOCK DN, KIMBER RJ, JUTTNER CA. High levels of circulating haemopoietic stem cells in very early remission from acute non-lymphoblastic leukaemia and their collection and cryopreservation. *Br J Haematol* 1984; 58:399-410.
- JUTTNER CA, TO LB, HO JQK, et al. Early lympho-hemopoietic recovery after autografting using peripheral blood stem cells in acute non-lymphoblastic leukemia. *Transplant Proc* 1988; 20:40-43.
- LOBO F, KESSINGER A, LANDMARK JD, et al. Addition of peripheral blood stem cells collected without mobilization techniques to transplanted autologous bone marrow did not hasten marrow recovery following myeloablative therapy. *Bone Marrow Transplant* 1991; 8:389-392.
- GIANNI AM, BREGNI M, SIENA S, VILLA S, SEIORELLI GA, RAVAGNANI F. Rapid and complete hemopoietic reconstitution following combined transplantation of autologous blood and bone marrow cells. A changing role of high dose chemoradiotherapy?. *Hematol Oncol* 1989; 7:139-148.
- MERINO J, SUBIRA ML, MARTIN ALGARRA S. Incubation of peripheral

blood mononuclear cells with rhIL-2 plus rhGM-CSF increases the rate of CD34+ and LAK cells in-vitro. Preliminary observations. *Int J Cell Cloning* 1992; 10:182-183.

17. SHWARTZBERG L, BIRCH R, BLANCO R, et al. Rapid and sustained hematopoietic reconstitution by peripheral blood stem cell infusion alone following high-dose chemotherapy. *Bone Marrow Transpl* 1993; 11:369-374.

18. BOIRON JM, MARIT G, FABERES C, et al. Collection of peripheral blood stem cells in multiple myeloma following single dose cyclophosphamide with and without recombinant granulocyte-macrophage colony-stimulating factor. *Bone Marrow Transpl* 1993; 12:45-55.

19. LASKY LC. Hematopoietic reconstitution using progenitors recovered from blood. *Transfusion* 1989; 29:552-557.

20. KOTASEK D, SHEPERD KM, SAGE RE, et al. Factors affecting blood stem cell collections following high dose cyclophosphamide mobilisation in lymphoma, myeloma and solid tumors. *Bone Marrow Transpl* 1992; 9:11-17.

21. GIANNI AM, BREGNI M, TARELLA C, STERN AC, PILERI A, BONADONA G. Granulocyte-macrophage colony-stimulating factor to harvest circulating haemopoietic stem cells for autotransplantation. *Lancet* 1989; 2:580-585.

22. SIENA S, BREGNI M, BRANDO B, et al. Flow cytometry for clinical estimation of circulating hematopoietic progenitors for autologous transplantation in cancer patients. *Blood* 1991; 77:400-409.

23. HOGHAUS S, GOLDSCHMIDT H, EHRHARDT R, HAAS R. Successful autografting following myeloablative conditioning therapy with blood stem cells

mobilized by chemotherapy plus rhG-CSF. *Exp Hematol* 1993; 21:508-511.

24. PETTINGELL R, MORGENSTERN GR, WOLL PJ, et al. Peripheral blood progenitor cell transplantation in lymphoma and leukemia using a single apheresis. *Blood* 1993; 82:3770-3777.

25. KESSINGER A, BIERMAN PJ, VOSE JM, ARMITAGE JD. High dose cyclophosphamide, carmustine and etoposide followed by autologous peripheral stem cell transplantation for patients with relapsed Hodgkin's disease. *Blood* 1991; 77:2322-2325.

26. KÖRBLING M, JUTTNER C, HENNON P, KESSINGER A. Autologous blastem cell versus bone marrow transplantation. *Bone Marrow Transplant* 1992; 10(S1):144.

27. MORTIMER J, HENDRICKS D, GOODNOUGH LT, ZUTTER M, FEARS C, BROWN RA. Pheresis of peripheral blood progenitor cells directed by CD34 counts. *Proc Amer Soc Clin Oncol* 1993; 12:467 (abstr n° 1629).

28. HAYLOCK DN, TO LB, DOWSE TL, JUTTNER CA, SIMMONS PJ. Ex-vivo expansion and maturation of peripheral blood CD34+ cells into the myeloid lineage. *Blood* 1992; 80:1405-1412.

29. AGLIETTA M, PIACIBELLO W, SANAVIO F, et al. Kinetics of human hemopoietic cells after in vivo administration of granulocyte-macrophage colony-stimulating factor. *J Clin Invest* 1989; 83:551-557.

30. TESTA U, PELOSI E, GABIANELLI M, et al. Cascade transactivation of growth factor receptors in early human hematopoiesis. *Blood* 1993; 81:1442-1456.

31. TAVASSOLI M, HARDY CL. Molecular basis of homing of intravenously transplanted stem cells to the bone marrow. *Blood* 1990; 76:1059-1070.

32. SHIOTA Y, MINGUELL J, ZANJANI ED, TAVASSOLI M. Induction of up-modulation of homing receptors in cloned hemopoietic progenitors by growth factors. *Bone Marrow Transplant* 1992; 9:123-127.

33. WILLIAMS GT, SMITH CA, SPOONER E, DEXTER TM, TAYLOR DR. Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. *Nature* 1990; 343:76-79.

34. TAVASSOLI M, KONNO M, SHIOTA Y, OMOTO E, MINGUELL JJ, ZANJANI ED. Enhancement of the grafting efficiency of transplanted marrow cells by preincubation with interleukin-3 and granulocyte-macrophage colony-stimulating-factor. *Blood* 1991; 77:1599-1606.

35. NAPARSTEK R, HARDAN Y, BEN-SHAHAR M, et al. Enhanced marrow recovery by short incubation of marrow allografts with human recombinant interleukin-3 and granulocyte-macrophage colony-stimulating factor. *Blood* 1992; 80:1673-1678.

36. SERKE S, SAUBERLICH S, HUHN D. A liquid culture method for the in-vitro growth of hemopoietic progenitor cells from normal human adult peripheral blood allowing for analysis by multiparameter flow-cytometry. *Eur J Hematol* 1991; 46:85-92.

37. BAIOCCHI G, SCAMBIA G, BENEDETTI P, et al. Autologous stem cell transplantation: Sequential production of hematopoietic cytokines underlying granulocyte recovery. *Cancer Res* 1993; 53:1297-1303.