

Monitoring Tumour Cell Purge by Long Term Marrow Culture in Acute Leukemia

MANAL EL-MASRY, M.D.* and TAREK HASHEM, M.D.**

The Departments of Clinical Pathology and Clinical Oncology**, Cairo*, Menofia** Universities*

ABSTRACT

Objective: Purging of leukemic cells from bone marrow harvested for autologous bone marrow transplantation (ABMT) remains a challenge. This work aimed at evaluating the efficacy of long-term marrow culture (LTMC) on purging leukemic progenitors in acute leukemia.

Design and methods: We planned to study the presence of immunoglobulin heavy (IgH) chain gene rearrangements by polymerase chain reaction (PCR) at diagnosis for bone marrow of 23 patients with acute leukemia. LTMC was performed only for patients who showed positive IgH chain gene monoclonality at diagnosis. The efficiency of purge was evaluated by PCR for monoclonal IgH chain gene on weekly basis of LTMC.

Results: Of the 23 studied cases, 18 (78.26%) showed positive clonal IgH chain gene at diagnosis. LTMC study showed that 6/18 (33.33%), 3/18 (16.67%), 7/18 (38.89%) and 2/18 (11.11%) underwent complete purging of the leukemic progenitors at the first, second, third and fourth weeks of culture, respectively. Follow up could be performed for 14 positive ALL cases after induction of remission; 12/14 (85.7%) showed minimal residual disease (MRD) while only two cases did not show MRD. Complete purging of the latter two cases by LTMC occurred on the second and third weeks of culture.

Conclusion: LTMC is a useful and successful method for leukemic cell purging. LTMC should be undertaken at initial diagnosis and on an individual basis. Each case should be dealt with solely to determine at which week of culture complete purging could be obtained for subsequent autologous grafting of the purged marrow.

Key Words: *Tumour cell purge - Long term marrow culture - Acute leukemia - IgH chain gene rearrangement.*

INTRODUCTION

High-dose chemotherapy with autologous peripheral blood or marrow hemopoietic cell support is an effective therapy for a number of malignancies such as patients with refractory acute leukemia. A major cause of treatment fail-

ure is disease relapse, which can result from resistant residual malignant cells in vivo or the infusion of contaminating tumour cells collected with the hemopoietic stem cells (HSC) [15,25].

LTMC in chronic myeloid leukemia (CML) and acute myeloblastic leukemia (AML) were shown to permit proliferation of normal hemopoietic progenitors while reducing leukemic progenitor cell numbers [13,20,21,28]. LTMC was also reported to be capable of sustaining residual normal hemopoietic proliferation and of damaging at the same time the leukemic population in marrow derived from patients with ALL [8,9].

To monitor MRD in LTMC, several techniques have been used including cytogenetic analysis and Southern blotting. These techniques, however, are either too insensitive or require many cells to detect MRD. PCR is a useful and highly sensitive technique for monitoring leukemic cell purging in various LTMC systems. IgH chain gene rearrangements have so far yielded information concerning tumour clonality and consequently can allow monitoring MRD in LTMC [10,26].

This work aimed at evaluating the efficacy of LTMC on purging leukemic progenitors in cases with acute leukemia on weekly basis using PCR technique to detect MRD among the clonogenic cells.

PATIENTS AND METHODS

Twenty-three patients with acute leukemia with age ranging from one to 17 years were enrolled in this study. The cell-lineage of almost all the leukemia cases (22/23) was determined

by immunophenotyping using flow cytometry (FCM) (Becton Dickinson) and antibodies against CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD13, CD19, CD20, CD22, CD24, CD33, CD34, CD45, as well as HLA DR, cytu and Igκ. One case was only diagnosed by morphology and cytochemistry-myeloperoxidase (MPO), acid phosphatase (ACP), periodic acid-Schiff (PAS), alpha naphthyl esterase [non-specific esterase (NSE)], naphthyl AS-D chloroacetate esterase [(CAE) specific esterase] - but not by immunophenotyping as marrow sample at diagnosis was only sufficient to perform the marrow culture. Also DNA index was calculated using FCM [19].

Bone marrow of all patients was studied at diagnosis for IgH chain gene rearrangements using PCR. Short-term marrow culture was performed at diagnosis to assess the clonogenic activity of marrow progenitors. LTMC was completed only for patients who showed positive monoclonal IgH gene rearrangements at diagnosis. The efficiency of tumour cell purge by LTMC was also evaluated by PCR for IgH monoclonality on weekly bases of culture.

Follow up to detect MRD could be performed for only 14 patients with ALL at day 43 post induction. Patients with ALL had induction therapy of prednisolone, vincristine, daunorubicin, asparaginase, VP-16, Ara-C and triple intrathecal chemotherapy for CNS prophylaxis. Patients were planned to receive high dose methotrexate consolidation chemotherapy to be followed by maintenance chemotherapy thereafter. Cranial irradiation was given for patients with CNS III aiming at 24 Gy/3w/15 ttt at a later stage.

Short-term marrow colony forming assay:

Bone marrow mononuclear cells (MNCs, 1×10^5) were isolated using lymphocyte separation medium (Gibco, Life Technologies, U.K), put in a sterile culture tube in 1 ml aliquot of Iscove's modified Dulbecco's medium 1x (IMDM, Sigma, Saint Fallavier, France) containing 10% fetal calf serum (FCS, Gibco) and 10^{-4} mol/l 2-mercaptoethanol (2-ME, Sigma). Culture was stimulated with 10 ng/ml interleukin-3 (IL-3, Amgen Inc, Thousand Oaks, Calif. U.S.A.), 10 ng/ml granulocyte-colony stimulating factor (G-CSF, Amgen), 10 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF, Amgen), 3 U/ml erythropoietin

(Epo, Amgen) and 50 ng/ml stem cell factor (SCF, Amgen). Pre-warmed Noble agar (0.3%) at 39-40°C was added to each culture tube, mixed rapidly and immediately pipetted in sterile 35-mm tissue culture dishes (Falcon, Subra, Toulouse, France). Progenitor cell growth was evaluated after 14 days incubation at 37°C and 5% CO₂ in humidified atmosphere [3,6].

LTMC assay:

Bone marrow MNCs (5×10^6) were isolated and seeded into 25 cm² tissue culture flask (Falcon) in a total volume of 10 ml of complete medium consisting of minimal essential medium (MEM)-Alpha medium 1x (Gibco) with 12.5% FCS, 12.5% horse serum (HS, Gibco), 2 mmol/l L-glutamine (Gibco), 10^{-4} mol/l 2-ME, 0.2 mmol/l inositol (Gibco), 20 μmol/l folic acid (Gibco) and 10^{-6} mol/l freshly dissolved hydrocortisone (Sigma). IL3 (10 ng/ml) and G-CSF (10 ng/ml) were added to the complete medium. Culture flasks were incubated at 33°C in a humidified atmosphere with 5% CO₂. They were fed weekly by replacement of half of the growth medium containing half of the non-adherent cells with fresh complete medium. After 5 weeks of culture, non-adherent cells and adherent cells harvested by trypsinization were pooled. The weekly-collected media containing the non-adherent cells as well as the adherent cells after the five week incubation were subjected to DNA extraction and PCR amplification for the monoclonal IgH gene rearrangement to assess purging of tumour cells [2,7]. LTMC was not subjected to in-vitro application of chemotherapeutic agents.

Detection of IgH monoclonality by PCR:

DNA extraction was performed by proteinase K digestion and salting out using standard methods [18]. The IgH gene rearrangement was amplified in a semi-nested two-round hot-start PCR (Perkin Elmer) using consensus primers (Table 1). Genomic DNA (100 ng) was added to the first round PCR mix. The first round of PCR involved 30 cycles of 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute with framework 3 primer (FR3A) and LJH primer. Two μl of the first round product were added to the second round reaction mix and amplified by 20 more cycles with the above cycling conditions using FR3A and VLJH primers. Both PCR rounds were performed in a 50 μl reaction containing 25 Taq PCR master mix (dNTP mix,

Taq DNA polymerase, PCR buffer containing 3 mmol/l Mg Cl₂) Qiagen) and completed to 50 µl by double-distilled water. If patients gave no amplified product with FR3A for the complementarity-determining region 3 (CDR3), then it was replaced by a framework 2 primer (FR2B) for the complementarity-determining region 2 (CDR2). The first gave a monoclonal band between 50 and 150 bp, while the latter gave a monoclonal band between 150 and 300 bp. PCR products were separated on 6% polyacrylamide gels and then stained with 0.3 g/ml ethidium bromide prior to visualization under UV light. Samples were scored as monoclonal band (M) at diagnosis if one or more distinct band(s) were observed in the presence or absence of any polyclonal background. Samples were scored as polyclonal (P) if only polyclonal PCR product was observed. The same criteria were used for marrow aspirates taken during induction, with the additional criterion that the monoclonal band(s) detected had to be of the same molecular size as that detected at diagnosis [11,24]. All PCR results were repeated at least twice to confirm the data.

RESULTS

This study included twenty-three patients with acute leukemia. They were 13 males (57%) and 10 females (43%) with male to female ratio 1.3:1. Their ages ranged from one to 17 years with a median age of 7 years.

A total number of 18/23 (78%) cases showed positive IgH gene monoclonality at initial diagnosis; 13/18 (72.2%) showed IgH monoclonal band at CDR3 between 50 and 150 bp using FR3A primer, while 5/18 (27.8%) showed IgH monoclonal band at CDR2 between 150 and 300 bp using FR2B primer (Table 3).

LTMC study showed that 6/18 (33.33%), 3/18 (16.67%), 7/18 (38.89%) and 2/18 (11.11%) underwent complete purging of the leukemic progenitors at the first, second, third and fourth weeks of culture, respectively, as evaluated by complete disappearance of the monoclonal IgH gene rearrangement band which was detected at initial diagnosis (Table 3, Fig. 1).

Follow up could be performed for 14 cases with ALL in complete clinical and hematological remission. A monoclonal IgH gene band of the same molecular size as that detected at diag-

nosis was observed in 12/14 (85.7%) patients on day 43 post induction, that might signify a minimal residual disease, while only two cases (14.3%) did not show any residual band post induction. Complete purging of the latter two cases by LTMC occurred on the second and third week of culture (Table 3).

Short-term culture results are shown in Tables (4,5). Correlation studies showed that presence of IgH gene monoclonal band at diagnosis was associated with decreased number of clusters detected by short-term culture ($r = -0.6, p = 0.007$) (Table 5). Significant correlation was observed between time of complete purging as compared to age, hemoglobin (HB) level, total leukocytic count (TLC), percentage of BM blast cells at initial diagnosis (BM1) and DNA index (DNAI) ($r = 0.6, -0.5, 0.74, 0.53, -0.6$), ($p = 0.02, 0.04, 0.001, 0.03, 0.03$), respectively (Table 6).

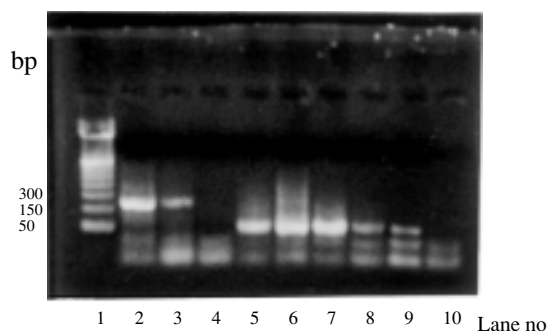


Fig. (1): IgH chain gene rearrangements in cases # 14 & 2 at diagnosis, post induction and at different intervals of LTMC: First lane: marker (ladder). Second lane: case no. 14 at diagnosis (positive M band at CDR2 between 150 and 300 bp). Third lane: case no. 14 after induction (positive M band at CDR2). Fourth lane: case no. 14 after first week of LTMC (negative M band at CDR2, complete purging). Fifth lane: case no. 2 at diagnosis (positive M band at CDR3 between 50 and 150 bp). Sixth lane: case no. 2 after induction (positive M band at CDR3). Seventh lane: case no. 2 after first week of LTMC (positive M band at CDR3). Eighth lane: case no. 2 after second week of LTMC (positive M band at CDR3). Ninth lane: case no. 2 after third week of LTMC (positive M band at CDR3). Tenth lane: case no. 2 after fourth week of LTMC (negative M band at CDR3, complete purging).

Table (1): Code and sequence of primers used for PCR analysis.

Code of primer	5' → 3' Sequence of primer
FR3A	ACACGGC(C/T)(G/C)TGTATTACTGT
LJH	TGAGGAGACGGTGACC
VLJH	GTGACCAGGGT(A/G/C/T) CCTTGGCCCCAG
FR2B	GTCTGCAGGC(C/T)(C/T)CCGG(A/G)AA (A/G)(A/G)GTCTGGAGTGG

Table (2): Immunophenotyping of studied cases.

Case No.	Marker																Cyt μ	Ig κ	Cell-lineage	
	CD 2	CD 3	CD 4	CD 5	CD 7	CD 8	CD 10	CD 13	CD 19	CD 20	CD 22	CD 24	CD 33	CD 34	CD 45	HLA DR				
1	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	Pro-B-ALL
2	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	cALL
3	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	cALL
4	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	cALL
5	-	+	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	Pre-T-ALL
6	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	+	-	Pre-B-ALL
7	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	+	-	Pre-B-ALL
8	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	+	Precursor B-ALL (transitional)
9	-	-	-	-	-	-	+	-	+	-	-	+	-	+	-	-	-	+	-	Pre-B-ALL
10	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	cALL
11	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	cALL
12	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	cALL
13	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	cALL
14	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	cALL
15	-	-	-	-	-	-	+	-	+	-	+	-	-	+	-	-	+	-	-	cALL
16	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	T-cell ALL (cortical)
17	-	+	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	T-with aberrant myeloid*
18	-	+	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	T-with aberrant myeloid*
19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ALL**
20	-	-	-	-	-	-	+	-	+	+	-	+	-	-	-	-	-	-	-	cALL
21	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	cALL
22	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	AML
23	-	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	AML

* Cases No. 17, 18: T-with aberrant myeloid = T-lineage ALL with aberrant expression of myeloid-associated marker.

** Case No. 19: ALL (mostly T-cell ALL) was only diagnosed by morphology and cytochemistry (MPO: -ve, ACP: +ve focal pattern, NSE: +ve discrete focal pattern, CAE: -ve, PAS: +ve discrete foci), as marrow sample at diagnosis was only sufficient to do marrow culture but not sufficient to do immunophenotyping.

Table (3): PCR results pre- and post-induction.

	PCR 1		PCR 2	Purging time (weeks)	Cell-lineage
	FR3A	FR2B			
1	Positive		Positive	4	Pro-B-ALL
2	Positive		Positive	4	cALL
3	Positive		Positive	3	cALL
4	Positive		Positive	3	cALL
5	Positive		Positive	3	Pre-t-ALL
6	Positive		Positive	3	Pre-B-ALL
7	Positive		Negative	3	Pre-B-ALL
8		Positive	Positive	3	Precursor B-ALL (transitional)
9	Positive		Positive	3	Pre-B-ALL
10		Positive	Positive	2	cALL
11		Positive	Negative	2	cALL
12	Positive		Positive	2	cALL
13	Positive		Positive	1	cALL
14		Positive	Positive	1	cALL
15	Positive			1	cALL
16	Positive			1	T-cell ALL (cortical)
17	Positive			1	T-with aberrant myeloid marker
18		Positive		1	T-with aberrant myeloid marker
19	Negative				ALL (mostly T-cell ALL)
20	Negative				cALL
21	Negative				cALL
22	Negative				AML
23	Negative				AML

PCR 1: PCR results pre-induction.

PCR 2: PCR results post-induction.

FR3A : Framework 3A primer for detection of CDR3.

FR2B : Framework 2B primer for detection of CDR2.

Table (4): Short-term culture results of 23 cases.

Variable	Mean	S.D.	Minimum	Maximum
Cluster (/10 ⁵ MNCs)	125.9	78.3	64	366
Colonies (/10 ⁵ MNCs)	51.71	70.68	9	290
Macroscopic colonies (/10 ⁵ MNCs)	8.6	20.4	0	94

Cluster: No. of cells < 50 cells.

Colony: No. of cells = or > 50 cells.

Macroscopic colony: size of colony by naked eye > 2 mm².

MNCs: Mononuclear cells.

Table (5): Correlation between different data and results of PCR at diagnosis of 23 cases.

	Age	Hb	TLC	Platelet	LDH	BM1	DNAI	Cluster	Colony	Macro
<i>r</i>	0.12	-0.04	0.07	0.17	0.06	0.03	-0.16	-0.6	-0.4	-0.08
<i>p</i>	0.65	0.86	0.76	0.46	0.82	0.89	0.57	0.007**	0.07	0.71

BM1 : BM blasts at initial diagnosis.

Macro: Macroscopic colonies.

Table (6): Correlation between different data and time of complete purging of 18 cases*.

	Age	Hb	TLC	Platelet	LDH	BM1	DNAI	Cluster	Colony	Macro
<i>r</i>	0.6	-0.5	0.74	-0.36	0.41	0.53	-0.6	-0.47	-0.42	-0.23
<i>p</i>	0.02*	0.04**	0.001***	0.18	0.2	0.03**	0.03**	0.06	0.11	0.4

* 18 acute leukemia cases that showed positive IgH chain gene monoclonality at diagnosis.

** Statistically significant ($p < 0.05$).

*** Statistically highly significant ($p < 0.005$).

DISCUSSION

The aim of this study was to determine the efficacy of LTMC on purging leukemic progenitors in cases with acute leukemia through the detection of a residual monoclonal IgH gene rearrangement among the clonogenic cells on weekly bases. It also aimed at studying the significance of their marrow clonogenic activity using short-term marrow culture as well as studying MRD after induction of remission.

Short-term marrow culture was performed for all the included 23 patients with acute leukemia. Correlation studies showed that presence of IgH gene monoclonal band at diagnosis was significantly associated with decreased number of clusters detected by short-term culture ($r = -0.6$, $p = 0.007$).

LTMC was completed for those who showed IgH gene rearrangement monoclonality at initial diagnosis (18 cases). LTMC study showed that 6/18, 3/18, 7/18 and 2/18 performed complete purging of the leukemic progenitors at the first, second, third and fourth weeks of culture, respectively, as evaluated by

complete disappearance of the monoclonal IgH gene rearrangement band which was detected at initial diagnosis. Thus, within four weeks of LTMC, all of the 18 cases that exhibited monoclonal IgH rearrangement at diagnosis showed complete purging of leukemic cells but at different stages of culture. These findings are in accordance with those reported by other authors who used the bcr/abl marker to monitor tumour cell purge by LTMC among patients with ALL showing positive bcr/abl fusion gene at diagnosis instead of monoclonal IgH gene rearrangement [23]. This could be explained by the proposal that leukemic progenitors have poor stromal adherence, while normal progenitors have good stromal adherence [12,16,27].

It was observed that LTMC might require longer time to purge BM of patients having clinical or laboratory findings that are considered as risk factors of relapse [22]. This means that monoclonal IgH gene rearrangement detected at initial diagnosis takes longer time to disappear from LTMC when certain risk factors are encountered. Significant correlation was observed between time of complete purging as

compared to age, HB level, TLC, percentage of BM blasts at diagnosis (BM1), DNA index ($p = 0.02, 0.04, 0.001, 0.03, 0.03$, respectively). This is in accordance with other authors who reported that BCR/ABL transcripts in ALL cases disappeared earlier in LTMC when initial leukemic infiltration of BM was less than 40%, while they disappeared later in LTMC when the initial leukemic infiltration of BM was more than 40%, besides the presence of other adverse prognostic factors [9]. These results are also in agreement with other authors who stated that a useful adjunct in risk assessment is the response to early treatment as measured by the rate of clearance of leukemic cells from blood or marrow or by the level of MRD after induction of clinical remission [1,22].

Follow up could be performed for only 14 cases with ALL after induction of remission. Monoclonal IgH gene rearrangement band of the same molecular size as that detected at diagnosis was persistent in 12/14 (85.7%) patients, while only 2/14 patients (14.3%) had no persistent monoclonal band after remission induction. Neither of the latter two cases showed complete purging on the first week of LTMC. They showed complete purging on the second and third week of LTMC. The incidence of MRD in our study is higher than that detected by Sykes et al., Gruhn et al. and Mayer et al. (30%, 27%, 61.5%, respectively) [24,14,17]. This difference could be due to the low number of included follow-up cases or due to less aggressive induction therapy of our cases. Six of the 12 positive follow up cases (50%) showed 3-4% blast cells in post-induction BM, while the other positive six cases (50%) showed 1% blast cells. This means that although they could be considered as being morphologically in remission, they had MRD that was detected by the monoclonal IgH gene rearrangement band. This is in accordance with the results reported by Gruhn et al. [14].

From this study, we can conclude that detection of IgH gene rearrangement monoclonality could be of value for management of leukemia in two ways. Its detection early in treatment as well as at the end of induction could identify patients who have risk of relapse; such patients may benefit from more intensive therapy. It can also be used to monitor tumour cell purge in LTMC. However, large prospective studies with BM sampling at fixed time points will be needed to determine whether a single assess-

ment of MRD levels in multiple follow-up samples during the first months of treatment will give better results [24].

We can also conclude that conditions prevailing in LTMC system may selectively deplete leukemic cells (as witnessed by disappearance of monoclonal IgH gene rearrangement that was detected at diagnosis), while promoting growth of residual normal progenitors. This suggests a possible extension of this therapeutic approach to some acute leukemia cases by means of 3-4 week in-vitro marrow culture to use it as a graft for ABMT. Autografting with purged in-vitro marrow culture can also be used as an alternative to marrow in-vitro treatment with chemotherapeutic agents. It is also possible that purging by LTMC can be used as a prognostic factor for ABMT, as evidenced by two studies that suggested that absence of tumour cells after purging correlated negatively with incidence of relapse [4,27]. For patients whose marrow can not be completely purged of tumour cells, post-transplantation therapy may be initiated early to prevent the higher relapse rate. As patients would have received high-dose chemotherapy for the transplantation, other forms for therapeutic modalities such as vaccine or cell-based immunotherapy might be employed [4]. For those patients whose marrow is purged free of tumour cells, improved conditioning therapies and adjunctive immunotherapies may ultimately lead to the goal of greater cure rates. It seems obvious that it is better to infuse a tumour-free marrow than one contaminated with tumour cells [5].

REFERENCES

- 1- Bigi G., Vanoli M. and Pomati M.: Myeloid differentiation in long term culture of human peripheral blood. *Exp. Hematol.*, 28 (12): 150-1509, 2000.
- 2- Blair A. and Sutherland H.: Primitive acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo lack surface expression of c-kit (CD17). *Exp. Hematol.*, 28 (6): 361-373, 2000.
- 3- Bradely T. and Metcalf D.: The growth of mouse bone marrow cells in vitro. *Aust. J. Exp. Biol. Med. Sci.*, 44: 287-284, 1966.
- 4- Bruserud O., Gjertsen B., Foss B. and Huang T.: New strategies in the treatment of acute myelogenous leukemia (AML): in vitro culture of AML cells-the present use in experimental studies and the possible importance for future therapeutic approaches. *Stem. Cells*, 19 (1): 1-11, 2001.
- 5- Bruserud O., Gjertsen B. and Von Volkman H.: In vi-

- tro culture of human acute myelogenous leukemia (AML) cells in serum-free media: studies of native AML blasts and AML cell lines. *J. Hematother. Stem. Cell Res.*, 9 (6): 923-932, 2000.
- 6- Cesana C., Carlo-Stella C., Mangoni L., Regazzi E., Garau D., Sammarelli G., Caramatti C., Almici C. and Rizzoli V.: In vitro growth of mobilized peripheral blood progenitor cells is significantly enhanced by stem cell factor. *Stem. Cells*, 15: 207-213, 1997.
 - 7- Dexter T., Allen T. and Lajtha L.: Conditions controlling the proliferation of haemopoietic stem cell in vitro. *J. Cell Physiol.*, 91: 335-341, 1977.
 - 8- Dormady S., Bashayan O., Dougherty R., Zhang X. and Basch R.: Immortalized multipotential mesenchymal cells and the hematopoietic microenvironment. *J. Hematother. Stem. Cells Res.*, 10 (1): 125-140, 2001.
 - 9- Espinoza-Hernandez L., Cruz-Rico J., Benitez-Aranda H., Martinez-Jaramillo G., Rodriguez-Zepeda M., Velez-Ruelas M. and Mayani H.: In vitro characterization of the hematopoietic system in pediatric patients with acute lymphoblastic leukemia. *Leuk. Res.*, 25 (4): 295-303, 2001.
 - 10- Fabrega S., Laporte J. and Giarratana M.: Polymerase chain reaction: a method for monitoring tumor cell purge by long-term culture in BCR/ABL positive acute lymphoblastic leukemia. *Bone Marrow Transplant.*, 11: 169-173, 1993.
 - 11- Galimberti S., Brizi F. and Mameli M.: An advantageous method to evaluate IgH rearrangement and its role in minimal residual disease detection. *Leuk. Res.*, 23: 921-929, 1999.
 - 12- Ghaffari S., Dougherty G., Eaves A. and Eaves C.: Diverse effects of anti-CD44 antibodies on the stromal cell-mediated support of normal but not leukemic (CML) haemopoiesis in vitro. *Br. J. Haematol.*, 97: 22-28, 1997.
 - 13- Guan Y. and Hogge D.: Proliferative status of primitive hematopoietic progenitors from patients with acute myelogenous leukemia (AML). *Leukemia.*, 2135-2141, 2000.
 - 14- Gruhn B., Hongeng S. and Yi H.: Minimal residual disease after intensive chemotherapy in childhood acute lymphoblastic leukemia predicts outcome. *Leukemia.*, 12 (5): 675-681, 1998.
 - 15- Hanazono Y., Terao K. and Ozawa K.: Gee transfer into nonhuman primate hematopoietic stem cells: implications for gene therapy. *Stem. Cells*, 19 (1): 12-23, 2001.
 - 16- Hidalgo A., Sanz-Rodriguez F., Rodriguez-Fernandez J., Albella B., Blaya C., Wright N., Cabandas C., Prosper F., Gutierrez-Ramos J. and Teixido J.: Chemokine stromal cell-derived factor-1 alpha modulates VLA-4 integrin dependent adhesion to fibronectin and VCAM-1 on bone marrow hematopoietic progenitor cells. *Exp. Hematol.*, 29 (3): 345-355, 2001.
 - 17- Mayer S., Giamelli J. and Sandoval C.: Quantification of leukemia clone-specific antigen gene rearrangements by a single step PCR and fluorescence-based detection method. *Leukemia.*, 13: 1843-1852, 1999.
 - 18- Miller S., Dykes D. and Polesky H.: A simple salting-out procedure for extracting DNA from human nucleated cells. *Nucleic Acid. Res.*, 16: 1215-1219, 1988.
 - 19- Neale G., Coustan-Smith E. and Pan Q.: Tandem application of flow cytometry and polymerase chain reaction for comprehensive detection of minimal residual disease in childhood acute lymphoblastic leukemia. *Leukemia.*, 13: 1221-1226, 1999.
 - 20- Pettengel R., Shido K. and Kanz L.: Preferential expansion of hemopoietic progenitor cells versus acute myeloid leukemia cells ex vivo. *Blood*, 86: 145-152, 1995.
 - 21- Podesta M., Piaggio G., Sessarego M., Pitto A., Benvenuto F., Vassallo F., Fugazza G., Carella A. and Frassoni F.: Spontaneous exodus of high numbers of normal early progenitor cells (Ph-negative LTC-IC) in the peripheral blood of patients with chronic myeloid leukemia at the beginning of the disease. *Br. J. Haematol.*, 97: 94-98, 1997.
 - 22- Radich J.: Clinical applicability of the evaluation of minimal residual disease in acute leukemia. *Curr. Op. Oncol.*, 12: 36-40, 2000.
 - 23- Spencer A., Yan X. and Chase A.: BCR-ABL-positive lymphoblastoid cells display limited proliferative capacity under in vitro culture conditions. *Br. J. Haematol.*, 94: 654-658, 1996.
 - 24- Sykes P., Snell L., Brisco M., Neoh S., Hughes E., Dolman G., Peng L., Bennett A., Toogood I. and Morely A.: The use of monoclonal gene rearrangement for detection of minimal residual disease in acute lymphoblastic leukemia of childhood. *Leukemia*, 11: 153-158, 1997.
 - 25- Vafai D., Vredenburgh J. and Ball E.: Purging of contaminating tumour cells. In: Ball E.D., Lister J. and Law P. (eds). *Haemopoietic stem cell therapy*: Churchill Livingstone, 322-334, 2000.
 - 26- Van Dogen J., Seriu T. and Panzer-Grumayer E.: Prognostic value of minimal residual disease in acute lymphoblastic leukemia in childhood. *Lancet.*, 352: 1731-1738, 1998.
 - 27- Van Hennik P., Breems D., Kusadasi N., Slaper-Cortenbach J., Van den Berg H., Van der Lelie H., Schipperus M., Cornelissen J. and Ploemacher R.: Stroma-supported progenitor production as a prognostic tool for graft failure following autologous stem cell transplantation. *Br. J. Haematol.*, 111 (2): 674-684, 2000.
 - 28- Verfaillie C., Bhatia R. and Miller W.: BCR/ABL-negative primitive progenitors suitable for transplantation can be selected from the marrow of most early-chronic phase but not accelerated-phase chronic myelogenous leukemia patients. *Blood*, 87: 4770-4779, 1996.