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Repeated High-dose Cyclophosphamide, BCNU and VP-16-213 and Autologous Bone Marrow Transplantation in Adult Acute Lymphocytic Leukemia in First Remission*

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Abstract—*In adult acute lymphocytic leukemia (ALL) cure is rare. The purpose of this study was to try to improve remission duration and survival by administration of two courses of high-dose chemotherapy, each followed by autologous bone marrow rescue, in first remission. Chemotherapy consisted of cyclophosphamide, BCNU and VP-16-213. Rescue bone marrow was fractionated over a discontinuous albumin gradient to minimize contamination with leukemic cells. Fourteen patients entered the study. Median total remission duration was* 14 months. *Three patients relapsed after one course of treatment. Five patients relapsed after the second course. Four patients died after the second course and two patients remain alive and well in unmaintained remission, with a total remission duration of* 42+ *and* 47+ months. *It is concluded that this regimen is toxic but, with careful selection of patients, may lead to long-term unmaintained remissions.*

INTRODUCTION

OF ALL patients over the age of 15 yr suffering from acute leukemia 20% have acute lymphoblastic leukemia (ALL), acute undifferentiated leukemia (AUL) or lymphoma-cell leukemia (LL) [1]. In adult ALL the median time of remission duration rarely exceeds 24 months [2, 3]. Improved results have recently been reported by Schauer *et al.* [4] and Clarkson *et al.* [5], whose patients achieved a median remission duration of 30 months. Continuing relapse is seen on survival curves, indicating that the prospect for cure is limited. The concept of intensification therapy in leukemia has been advocated by Jacquillat *et al.* [6] and Bodey *et al.* [7], whose studies show prolongation of complete remission (CR) in patients who received high-dose chemotherapy early or late in remission. Another form of high-dose cytoreductive therapy is the use of chemotherapy and total-body irradiation in conjunction with bone marrow transplantation, studied by Thomas *et al.* [8]. Allogeneic bone marrow transplantation in second-remission ALL leads to a median remission duration of 9 months [8–10]. However, only 25% of the patients have a suitable donor and the survival after allogeneic bone marrow transplantation is also limited by occurrence of graft-vs-host disease (GvHD), interstitial pneumonia and immunodeficiency [11]. As an alternative, autologous bone marrow transplantation (ABMT) has been explored in relapsed leukemia [12–16]. In our study, using piperazinedione followed by total-body irradiation (TBI), remissions lasted up to 14 months, but eventually all patients relapsed [15]. Theoretically, relapse could have been caused either by leukemic cells that escaped the cytoreductive treatment prior to autologous transplantation or by leukemic cells present in the reinfused marrow. These dismal results, however, do not necessarily predict for a similar clinical outcome in remission. Hervé *et al.* reported two potential long-term disease-free survivors with

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acute myelogenous leukemia (AML) who received ABMT in first remission, after high-dose chemotherapy consisting of 6-thioguanine, cytarabine, lomustine and cyclophosphamide [16]. Unpublished data from Seattle suggest a similar outcome in AML following high-dose cyclophosphamide and total-body irradiation plus ABMT, with 100 days of intermittent methothrexate after transplant [Thomas, personal communication]. In ALL there is particular interest in ABMT in remission with the use of bone marrow treated with either anti-CALLA or anti-Leu-1 monoclonal antibody [17, 18]. However, in view of the potential positive data with ABMT in AML in first remission, some data on the natural history of the treatment of ALL in first remission with ABMT without monoclonal antibody treatment is warranted.

As cytoreductive regimen we chose high-dose combination chemotherapy consisting of cyclophosphamide, BCNU and VP-16-213 (CBV), which in our hands had shown considerable activity in relapsed leukemia refractory to conventional treatment [19]. CBV did cause significant prolonged myelosuppression, and therefore we felt autologous bone marrow rescue was necessary.

The collected bone marrow was fractionated on a discontinuous albumin gradient in an attempt to separate stem cells, as measured by the CFU-C assay, from the majority of residual leukemic cells. *In vitro* separation results showed a 55–90% reduction of leukemic cells [20].

MATERIALS AND METHODS

Patient characteristics

Fourteen patients, seven males and seven females, were entered in this program. The median age of these patients was 21 yr, with a range of 16–49 yr. The histopathologic diagnosis was acute lymphocytic leukemia in ten patients, acute undifferentiated leukemia in two patients and convoluted cell lymphoma leukemia in two patients. In 11 patients cell surface markers were determined. Seven patients had null cell leukemia and four patients were positive for T-cell markers. The four patients with positive T-cell markers presented with extramedullary disease as well as bone marrow involvement (three with mediastinal lymphadenopathy and one with mediastinal lymphadenopathy and bilateral axillary nodes as well as bilateral breast involvement). The patients had received uniform remission-induction treatment with a combination of cyclophosphamide (1000 mg/m^2), rubidazone (200 mg/m^2), vincristine (2 mg), prednisone (100 mg × 5 days), L-asparaginase (20,000 units/m^2) and methotrexate (120 mg/m^2). After one course of consolidation with the same drugs patients received maintenance chemotherapy with one course of cytarabine plus 6-thioguanine followed by one course of cyclophosphamide, rubidazone, vincristine and prednisone. Then followed the transplant regimen. All patients signed informed consent agreeing to bone marrow aspirations as well as to the administration of chemotherapy followed by bone marrow rescue.

Preparation of bone marrow cell suspensions

Bone marrow cells were collected when patients were in complete remission (CR) for 2–3 months (after their first maintenance course) and upon recovery from the first autologous transplant. Multiple bone marrow aspirations were carried out under general anesthesia from posterior iliac crests. An average of 1500 ml of bone marrow cells were harvested and suspended in Hank's balanced salt solution (HBSS) with preservative-free heparin.

Fractionation of bone marrow cell suspensions by discontinuous albumin gradients

This method has been described in detail [21]. In short, erythrocyte-poor marrow cells were suspended in 17% albumin solution (bovine serum albumin (BSA), fraction V, Sigma) and pipetted on top of denser albumin layers consisting of 21, 23 and 25% albumin solutions of defined osmolarity. These fractions were then centrifuged for 30 min at 10°C in an International centrifuge at 2000 revs/min, corresponding to 1000 ***g*** at the bottom of the tube. After centrifugation distinct layers of cells were visible in the gradient near the interfaces between the different albumin layers. The cell fraction between the 17 and 21% BSA layers has been labelled fraction 1+2, between 21 and 23% BSA fraction 3, and so on to fraction 5 at the bottom of the tube. Each fraction was then collected and diluted with HBSS. Samples of each fraction were studied morphologically by light microscopy as well as electron microscopy for the presence of leukemic cells [22].

Bone marrow storage and thawing

The different bone marrow fractions were stored according to the technique described by Schaefer *et al.* [23]. Dimethylsulfoxide (DMSO) and fetal bovine serum (FBS) were added to the HBSS-suspended bone marrow cells (final concentration of DMSO 10%, FBS 20%). The cells were transferred to 5 ml polypropylene ampules and cooled at 1°C/min to -40°C using a Cryoson automatic controlled freezer. After rapid cooling from -40 to -80°C, the cells were stored in liquid nitrogen at -192°C. At the time of transplantation

the ampules were thawed rapidly in a 50°C waterbath. Immediately after thawing, the cells were slowly diluted with HBSS until the original volume was diluted 10-fold. After step-wise dilution the cells were centrifuged, resuspended in HBSS and filtered through G2 glass filters (Jena glass, pore size 40–80 μm). Viability was tested by the CFC-GM assay. All patients were premedicated intravenously with 25 mg diphenhydramin hydrochloride (Benadryl) and 250 mg methylprednisolone (Solu-medrol).

Conditioning of the patient

The high-dose combination chemotherapy consisted on cyclophosphamide, BCNU and VP-16-213 (CBV), and was given upon recovery of the second maintenance treatment. Bone marrow was reinfused 2–3 days later. The treatment schedule is outlined in Table 1. Cyclophosphamide was given daily at a dose of 1.5 gm/m^2 in 1000 ml of dextrose for 3 days; BCNU was given as a single dose of 300 mg/m^2 in 250 ml dextrose over 1 hr on day 1, and VP-16-213 was given twice daily at a dose of 75 mg/m^2 per injection in 250 ml normal saline over 2 hr for 4 days, to a total dose of 600 mg/m^2. Upon recovery from the first course the procedure of multiple bone marrow aspirations was repeated. Bone marrow cells were fractionated and stored and the second course of cyclophosphamide, BCNU and VP-16-213 was administered at exactly the same doses as the first, with a second rescue using the bone marrow stored after recovery from the first course. The average time between courses 1 and 2 was 8 weeks.

Table 1. *Treatment schedule of CBV with autologous bone marrow rescue*

	Day						
	1	2	3	4	5	6	7
Cyclophosphamide, 1.5 g/m^2	X	X	X				
BCNU, 300 mg/m^2	X						
VP-16-213, 150 mg/m^2	X	X	X	X			
Bone marrow rescue							X

Supportive care

Patients were treated in an open hospital ward and received prophylactic antibiotic combinations consisting of oral trimethoprim-sulfamethoxazole (Bactrim®), nystatin and intravenous tobramycin as soon as the granulocyte count fell below 0.5 × 10^9/l.Whenever fever occurred while the patient was being treated with prophylactic antibiotics the treatment was changed to intravenous cefoxitin and ticarcillin. If fungal infection was suspected, miconazole or amphotericin B was added. When fever did not respond within 2–3 days, leukocyte transfusions from related family members were given until defervescence or recovery of granulocytes. Single-donor platelet transfusions were given prophylactically when platelet counts were less than 20 × 10^9/l. All blood products were irradiated with 2500 rad prior to transfusion.

Central nervous system prophylaxis

Intrathecally all patients received methotrexate (20 mg) alternating with ara-C (100 mg) on a monthly basis for a total of 12 months.

RESULTS

Patient characteristics, remission duration and outcome are shown in Table 2. Median remission duration prior to transplantation was 3 months, with a range of 3–8 months. In patient 12 bone marrow transplantation was postponed because of occurrence of superficial phlebitis in the right leg. Median remission duration after transplantation was 11 months, with a range of 2–43+ months. Of the 14 patients treated, two (Nos 13 and 14) remain in CR at 43+ and 39+ months post-transplantation. Patient Nos 1, 2 and 3 relapsed after the first course of CBV (on days 26, 62 and 63 respectively). They were considered resistant to chemotherapy and were taken off protocol. Three patients died shortly after the second course of CBV of complications: patient 9 developed an α-*Streptococcus* septicemia on day 8 after the second transplant and died on day 12 from pulmonary hemorrhage, secondary to diffuse intravascular coagulation (DIC). Patient 10 died from generalized Herpes zoster infection on day 35 after second transplant, and patient 11 died on day 51 after the second bone marrow transplant of *E. coli* septicemia and suspected fungal infection without evidence of engraftment. Four patients (Nos 4, 5, 6 and 7) relapsed in their bone marrow respectively at 1, 4, 5 and 9 months after their second transplant (4, 10, 11 and 12 months after their first transplant). Patient 7 was successfully reinduced into CR with piperazinedione (50 mg/m^2) and total-body irradiation, followed by an allogeneic bone marrow transplant from a sibling, but subsequently relapsed again. She is currently alive and well in her fourth CR. Patient 8 suffered a CNS relapse at 25 months after the second transplant and subsequently died of meningitis due to a infected Ommaya shunt. At time of death bone marrow and CNS were free of leukemia. Patient 12 died 11 months after the second transplant from intractable heart failure due to cardiomyopathy. This patient had received more anthracyclines than the other patients, with a total dose of 880 mg/m^2 of rubidazone during maintenance treatment before transplantation. His entrance into the study had been delayed

Table 2. Patient characteristics and outcome

Patient No.	Age	Sex	Diagnosis	Surface markers	Time from CR to CBV (months)	CR duration (months) from onset of CBV	Outcome
1	18	F	ALL	not done	3	1	†relapse
2	49	F	ALL	not done	3	2	†relapse
3	17	M	ALL	null cell	3	2	†relapse
4	18	F	ALL	null cell	3	4	†relapse
5	16	F	ALL	null cell	4	10	†relapse
6	23	M	AUL	T cell	3	11	†relapse
7	35	F	ALL	not done	3½	12	alive, 4th CR
8	16	M	ALL	T cell	3	28	†CNS relapse
9	18	M	LL	T cell	3	4+	†infection
10	26	M	ALL	null cell	3½	7+	†infection
11	18	F	ALL	null cell	3½	11+	†aplasia
12	41	M	AUL	null cell	8	16+	†cardiomyopathy
13	30	F	LL	T cell	3	39+	alive
14	25	M	ALL	null cell	4	43+	alive

because of thrombophlebitis of the right leg. Also, he was the oldest patient to receive two transplants.

Toxicity

With three patients relapsing after the first course, the total number of courses given to these 14 patients was 25. Nine patients developed a fever of unknown origin during myelosuppression; four patients developed bacterial sepsis, with a fatal outcome in two: one patient died of generalized Herpes zoster and one patient had suspected fungal infection. Both fatal infections occurred during the second course, indicating possible cumulative toxicity. Except for patient 9, who developed DIC secondary *α-Streptococcus* sepsis, no hemorrhage occurred. The median number of prophylactic platelet transfusions given was three. The median duration of suppression of neutrophil counts below $0.5 \times 10^9/l$ was 17 days, ranging from 10 to 27 days. Full hemopoietic recovery was obtained within 1 month after transplant in most cases. When the duration of myelosuppression following the first and second courses was compared, recovery after the second course was more protracted, with an average delay of 6 days in the second course (Table 3).

Table 3. Hemopoietic recovery after the first and second courses of CBV with autologous bone marrow transplant

	1st course	2nd course
500 granulocytes/μl	19*	25
1000 granulocytes/μl	24	30
20,000 platelets/μl	15	23
50,000 platelets/μl	20	25
100,000 platelets/μl	24	28

*Median days after infusion of autologous bone marrow.

Rescue bone marrow data

Upon collection and fractionation, all fractions were studied for the presence of leukemic cells. In three patients (Nos 1, 3 and 8) leukemic cells were found to be present in the light-density fractions (fractions 1 + 2), and in one patient (No. 8) cells were also present in fraction 3, while no leukemic cells were identified before the fractionation procedure. In fractions 4 and 5 the presence of leukemic cells could not be demonstrated. All patients received fractions 3, 4 and 5 as bone marrow rescue except patient No. 8, in whom leukemic cells were present in fraction 3. He received fractions 4 and 5 only. The total cell number administered to the patients varied from 0.25×10^8 to 2.9×10^8/kg body wt (median 1.1×10^8/kg body wt). All reinfused marrows were positive for *in vitro* growth of precursor cells (CFC-GM). There was no significant difference between the number of CFC-GM obtained from marrow collected before and after the first CBV treatment. Bone marrow aspirations taken on day 7 after transplant showed evidence of engraftment (morphologic criteria: presence of early red cell and/or myeloid precursors) in all patients except one (No. 11). This patient did receive a total of 1.2×10^8 bone marrow cells/kg body wt, with adequate CFC-GM growth, but died with a hypocellular bone marrow on day 51 after the second transplant.

DISCUSSION

The role of autologous bone marrow transplantation in acute leukemia is still under investigation, although this approach was first proposed in 1959 [12]. In this article we describe the results of a pilot study in adult ALL in first remission using two consecutive courses of high-dose combination chemotherapy, each followed by rescue with fractionated autologous marrow. In most studies conditioning regimens for bone

marrow transplantation in leukemia consist of ablative chemotherapy plus supralethal irradiation. High-dose chemotherapy without TBI has been used in autologous [16, 19; Thomas, personal communication] as well as allogeneic bone marrow transplantation [24, 25]. The choice of the chemotherapeutic regimen was based on our previous promising results in refractory ALL [19], where a 33% CR rate was observed. High-dose combination chemotherapy, although probably less cytoreductive than total-body irradiation, does result in prolonged aplasia, which can be minimized by autologous bone marrow infusion [25]. Because of the activity of CBV, relapse early after transplantation would be unexpected, unless leukemic cells did contaminate the infused marrow and were easily transplantable. Leukemic cells differ from normal hemopoietic stem cells as detectable by physical [27–31], chemical [32, 33] and immunological [34–37] methods. In this study we used physical separation by fractionation over a discontinuous albumin density gradient, which in our hands results in a 50–90% reduction of leukemic cells [20]. The three relapses early after the first course are worrisome in that they could possibly have been caused by leukemic cells still present after the fractionation procedure. In fact, two of the early relapses did have morphologically identifiable leukemic cells before transplantation (in the light-density fractions that were not reinfused). Maybe other methods to separate leukemic cells from normal stem cells are necessary, such as *in vitro* chemotherapy [39] or immunological separation using monoclonal antibodies [17, 18].

The principle of doing the second transplant, using marrow collected after the first course, was the assumption that if ALL cells were transplanted, the major residual disease after the first course would be the transplanted cells (CBV hopefully eliminating most *in vivo* residual disease) and marrow collection would mean an almost 2 log reduction again: if we assume that the total amount of leukemic cells present in remission is 1×10^7 cells, first marrow collection would contain 2×10^5 cells, because approximately 2% of the total body reserve of bone marrow is collected for storage. Assuming a favorable 1 log reduction by density fractionation, 2×10^4 cells would be reinfused. The rate of proliferation of the leukemic cell population early after transplantation is unknown, and may vary among the patients. The average time from first transplant to second storage was 2 months. Assuming a doubling time of 5–6 days and a growth fraction of 10%, the 2×10^4 reinfused leukemic cells would grow out to 2×10^6 at the time of the second collection and 2% of this, or 4×10^4, would be in the stored marrow, with gradient fractionation lowering the number to 4×10^3 leukemic cells reinfused at second transplant.

Maybe by a double transplant residual *in vitro* leukemic cells would be eliminated and the small percentage of reinfused leukemic cells at time of second transplant could be eliminated by the patients themselves. If these theoretical considerations were correct, then we could expect the patients who did receive two transplants (11 total) to have long-term unmaintained control of leukemia.

Unfortunately four patients died of complications related to their second transplant (patients 9, 10, 11 and 12), so only seven patients are available to test such a hypothesis. These patients had remission durations post-CBV of 4, 10, 11, 12, 28, 39+ and 43+ months. This is not significantly different from results achieved with conventional chemotherapy, but with two long-term survivors—one of which is a T-cell lymphoma leukemia—maybe there is a suggestion that if marrow is not heavily contaminated prolonged disease-free survival is possible with such an approach, if it can be done safely.

The hemopoietic recovery after the first and second courses was different. Time to normalization of peripheral blood values after the first course is comparable to that observed by Gorin *et al.*, using the TACC regimen [25], and Graw *et al.*, using the BACT regimen [24]. The fact that time to recovery was longer after the second course than after the first could mean that marrow collected after high-dose chemotherapy has less potential to repopulate the bone marrow, or that the hemopoietic microenvironment has been affected.

Our conclusion is that the results of this phase II study are comparable to conventional regimens, with the advantage that the remissions are unmaintained. However, the toxicity in this patient group was unacceptable, with 4/14 patients dying in CR. As all problems occurred after the second transplants, we conclude that there was cumulative hematopoietic and, possibly, cardiac toxicity. Careful selection may overcome some of the treatment-related deaths, and collection of all rescue bone marrow before the first course of CBV may prevent engraftment failures. Monoclonal antibodies may help in future detection and elimination of residual leukemic cells in the stored autologous marrow, and we plan to evaluate the use of anti-leukemic monoclonal antibodies as *in vitro* treatment of autologous bone marrow.

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